BIOCHEMICAL MECHANISMS MEDIATING MORPHOLOGICAL PLASTICITY OF ASTROGLIAL CELLS IN CULTURE

By

Katrina Dee Ramsell

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ABSTRACT

BIOCHEMICAL MECHANISMS MEDIATING MORPHOLOGICAL PLASTICITY OF ASTROGLIAL CELLS IN CULTURE

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Activation of the hypothalamo-neurohypophysial system, during dehydration, parturition, or lactation has previously been shown to be associated with plasticity of ultrastructural relations between the magnocellular neurosecretory axon terminals and pituicytes, the resident neurohypophysial astroglia. Cultured pituicytes, derived from adult rat neurohypophyses, have previously been reported to change from a flat, epithelial-like form to a stellate, process-bearing form when incubated in a serum-free medium containing a β-adrenergic agonist. The studies presented here were designed to investigate how potential modulators of pituicyte ultrastructural plasticity *in vivo* could affect the morphology of pituicytes in culture and what biochemical mechanisms mediate these effects.

These studies demonstrate that β-adrenoreceptor activation by isoproterenol, direct activation of adenylate cyclase by forskolin, and 8-bromo cAMP induce stellation of cultured pituicytes, indicating that increased intracellular cAMP concentration mediates stellation of pituicytes in culture. Paradoxical effects were observed with the protein kinase A inhibitors H-7, H-89, and Rp-cAMPs, in that these agents induced stellation and did not inhibit cAMP-mediated stellation. Nitric oxide donors (sodium nitroprusside and SIN-1), atrial natriuretic peptide, and 8-bromo cGMP also induce stellation of cultured pituicytes, indicating that increased intracellular cGMP concentration mediates stellation of cultured pituicytes.

Serum attenuates and reverses cAMP-mediated stellation of cultured pituicytes, and serum does not exert its effects by acting via an inhibitory G-protein nor by increasing phosphodiesterase activity, but rather by acting downstream of changes in intracellular cAMP concentration. Forskolin- and serum-induced changes in pituicyte morphology occur independently of changes in intracellular Ca^{+2} concentration. The tyrosine kinase inhibitor genistein attenuates the effects of serum on forskolin-induced stellation and on reversal of stellation of cultured pituicytes and of RG-2 astroglial cells. Data presented here suggest that agents which increase intracellular cyclic nucleotide concentration can induce stellation of cultured pituicytes, that serum can activate a tyrosine kinase to maintain astroglial cells in a non-stellate morphology, and that there is an association between the tyrosyl-phosphorylation status of the presumed cytoskeletal proteins focal adhesion kinase and paxillin and the cellular morphology of RG-2 astroglial cells. Results from these studies suggest that intracellular cyclic nucleotide concentration and serum may play critical roles in regulating astroglial plasticity *in vivo*.

To all the rats who gave their lives to make this work possible.
Hopefully their sacrifice will serve to better the lives of humans and animals alike.

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I would like to express my sincere appreciation to Dr. Peter Cobbett for providing me the opportunity to work in his laboratory. His guidance and advice has helped me to grow on an academic level, and I am grateful for the time and patience that he has provided me over the course of my graduate career. I would also like to thank Dr. Peggy Contreras, Dr. James Galligan, and Dr. Cheryl Sisk. Their advice and critical review of this dissertation have been extremely helpful. I would also like to acknowledge contributions made by Dr. Diane Matesic, Dr. Stephanie Watts, and Dr. M. Sue Marty. And finally, best wishes to my lab partner Mike Hlubek.

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α-MSH	melanocyte stimulating hormone
ANP	atrial natriuretic peptide
ATP	adenosine triphosphate
C	celsius
Ca^{+2}	calcium ion
cAMP	cyclic adenosine 3',5'-monophosphate
cGMP	cyclic guanosine 3',5'-monophosphate
Cl	chlorine
cNOS	constitutive nitric oxide synthase
CNS	central nervous system
CO_2	carbon dioxide
Cy3	indocarbocyanine
DMEM	Dulbecco's modified essential medium
DMSO	dimethyl sulfoxide
EGTA	ethylene glycol-bis (β-aminoethyl ether) N,N,N,N'-teracidic acid
ERK	extracellular signal-regulated kinase
g	gram
GC	guanylate cyclase
GFAP	glial fibrillary acidic protein
Gi	inhibitory G-protein
GTP	guanine triphosphate
Gq	pertussis toxin insensitive G-protein
hr	hour
HBSS	HEPES buffered salt solution
HCl	hydrochloric acid
HEPES	[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HNS	hypothalamo-neurohypophysial system
Hz	hertz
IBMX	3-isobutylmethyl-1-xanthine
IF	intermediate filament protein
IgG	immunoglobulin
iNOS	inducible nitric oxide synthase
K^+	potassium ion
KDa	kilodalton
Kg	kilogram

Ki	inhibitory constant
l	liter
L-NAME	nitro^{G}-L-arginine methyl ester
LPA	lysophosphatidic acid
LY-83,583	6-anilo-5,8-quinolinedione
M	molar
MAP	mitogen activated protein
min	minute
ml	milliliter
mM	millimolar
MNC	magnocellular neuroendocrine cell
mm	millimeter
mOsmol	milliosmolar
MY-5445	1-(3-chlorophenylamino)-4-phenylphthalazine
Na	sodium
NADH	β-nicotinamide adenine dinucleotide, reduced
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced
NCS	newborn calf serum
Ni	nickel
nm	nanometer
NO	nitric oxide
NOS	nitric oxide synthase
PBS	phosphate buffered saline
PDE	phosphodiesterase
PKA	cyclic AMP-dependent protein kinase
PKG	protein kinase G
PLC	phospholipase C
PMA	phorbol-12-myristate-13-acetate
PMSF	phenylmethylsulfonyl fluoride
PTX	pertussis toxin
PVN	paraventricular nuclei
p125FAK	focal adhesion kinase
Rp-cAMPS	Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine
SDS	sodium dodecyl sulfate
sec	second
SFO	subfornical organ
SIN-1	3-morpholinosydnonimine
SNP	sodium nitroprusside
SON	supraoptic nucleus
u	units

INTRODUCTION

The largest class of non-excitable cells in the brain are the neuroglia, and among the most significant of these are the astroglia, or as they are more commonly termed, astrocytes. Although research has focused on the activities of neurons to explain brain function, it is becoming increasingly clear that astroglial cells play important and diverse roles in normal brain physiology, brain development, and in pathology of the central nervous system. Recent studies have indicated that astrocytes are plastic in shape and have a variety of regulatory functions, and the discovery of GFAP (glial fibrillary acidic protein), which is an intermediate filament protein unique to astrocytes (Eng *et al.*, 1971), has contributed to elucidating astrocyte structure and function.

Astrocytes are situated around neurons and neuronal processes in the brain and have been implicated in neuronal guidance, in trophic support of neurons, and in regulation of communication between neurons. They are involved in controlling local ion and amino acid concentrations, and they can promote (Matthiessen *et al.*, 1989) or inhibit (Liuzzi and Lasek, 1987) axonal regeneration. In addition, astrocytes synthesize a variety of substances, such as nerve growth factor and the extracellular matrix components laminin and fibronectin which serve to regulate neuronal activities (Kimelberg and Norenberg, 1989).

Astroglial cells exhibit a large variety of receptors on their surface for neuropeptides, amines, amino acids, eicosanoids, and a variety of growth factors (Krisch and Mentlein,

1994). Activation of these receptors can alter cyclic nucleotide concentration (Stone and John, 1991; Stone *et al.*, 1990) or utilize the phosphatidylinositol intracellular signaling pathway to affect intracellular calcium concentration and production of diacylglyerol (Murphy and Pearce, 1987). These second messengers can then mediate a cascade of events via the activation of specific protein kinases. Receptor-mediated responses of astrocytes to external stimuli include modification of ion channels, alteration of cell metabolism, synthesis and secretion of a variety of substances, uptake and clearance of neurotransmitters from intercellular clefts, and plastic remodeling of the astrocyte cytoskeleton (Krisch and Mentlein, 1994).

Ultrastructural plasticity of astroglial cells has been documented in several regions of the brain, including the cortex, cerebellum, hippocampus, and the hypothalamo-neurohypophysial system (HNS). Astroglial plasticity has also been demonstrated in primary cultured astrocytes and in astroglial cell lines. Agonists which induce cAMP formation in astrocytes, or agents which elevate cAMP directly, have been shown to cause changes in the phosphorylation of cytosolic proteins and can induce rearrangement of the actin cytoskeleton (Murphy and Pearce, 1987). Astroglial cells derived from the adult rat neurohypophysis, termed pituicytes, are similar to other astroglial cells in that they have an array of receptors and have been shown to exhibit morphological plasticity (Tweedle and Hatton, 1987; Bicknell *et al.*, 1989). Studies presented here have focused on biochemical pathways which are involved in regulating morphological plasticity of astroglial cells, and pituicytes have been used as a primary model to investigate biochemical mechanisms mediating astroglial plasticity.

Pituicytes are specialized astrocytic glial cells of the mammalian neurohypophysis, and they have a highly plastic relationship with neurohypophysial neurosecretory terminals. Under basal conditions, pituicytes have cytoplasmic and membranous material interposed between neurosecretory axon terminals as well as between axon terminals and the basal lamina. When the hypothalamo-neurohypophysial system is activated, for example during lactation or dehydration, pituicytes withdraw their cytoplasmic and membranous material from between the axon terminals and from between the axon terminals and the basal lamina (Tweedle and Hatton, 1980a, 1982, 1987). This allows increased apposition of the axon terminals with the basal lamina and may therefore allow increased hormone secretion into the systemic circulation via the fenestrated capillaries which course through the neurohypophysis.

In culture, pituicytes change morphologically from a flat, epithelial-like shape to a stellate, process-bearing form (Bicknell *et al.* 1989). Taking into consideration that cultured pituicytes are constrained to a two-dimensional substrate, morphological alterations observed *in vitro* are similar to those described following three-dimensional reconstruction of pituicyte ultrastructural changes *in situ* (Gregory *et al.*, 1982), and it is likely that morphological changes of pituicytes in culture represent the ultrastructural changes which occur *in vivo*. The significance of pituicyte stellation *in vitro* and of pituicyte cytoplasmic withdrawal *in vivo* is still unknown, but it is likely that withdrawal of pituicyte cytoplasmic material *in vivo* is instrumental in regulating hormone secretion from the neurohypophysis into the systemic circulation.

This work has focused on biochemical mechanisms mediating alterations in astroglial

morphology, and pituicytes, derived from the adult rat neurohypophysis, have served as a model for investigating signal transduction pathways which mediate astroglial plasticity. It is hoped that findings from pituicyte studies may serve to elucidate biochemical mechanisms mediating morphological plasticity of astroglial cells and brain plasticity in general. This investigation has important implications for a better understanding of brain functions, such as regulation of hormone secretion, and for understanding disease mechanisms which are at least partially due to astroglial malfunction, such as Huntington's Disease, Parkinson's Disease, and epilepsy (Kimelberg and Norenberg, 1989). Since ultrastructural changes occur within the HNS (in both the hypothalamus and neurohypophysis), and neurohypophysial-derived astrocytes are the main model for investigating astroglial plasticity in these studies, the structure and function of the HNS is described in detail. However, within these descriptions, the reader should focus on plasticity in the HNS with respect to biochemical mechanisms of plasticity in the HNS and the role of astrocytes in ultrastructural plasticity.

I. The Hypothalamo-Neurohypophysial System

The hypothalamo-neurohypophysial system (HNS) is a well established mammalian model system for studying neurosecretion, but cellular mechanisms underlying the plastic ultrastructural relations between the magnocellular neuroendocrine cells and adjacent hypothalamic and neurohypophysial astrocytes are poorly understood. Magnocellular neuroendocrine cell bodies of the anterior hypothalamus reside primarily in the supraoptic nuclei (SON) and magnocellular paraventricular nuclei (PVN) (Armstrong *et al.*, 1980), but they are also scattered throughout the anterior hypothalamus (Peterson, 1966). Early light

microscopic observations showed the disappearance of neurosecretory material from pituicytes when an animal was dehydrated, and this led to the misconception that pituicytes were responsible for the manufacture and release of neurohypophysial hormones (Fisher *et al.*, 1938; Gersh, 1939). This notion was later dispelled when Bargmann and Sharrer (1951) demonstrated that the "neurosecretory material" in the neurohypophysis was actually manufactured in the hypothalamic cell bodies and transported down axons to the neurohypophysis.

Magnocellular neuroendocrine cells (MNCs) of the PVN and SON have axons which collect in the neurohypophysial tract and project to the neurohypophysis via the internal zone of the median eminence (Figure 1). These axons branch and terminate in the neurohypophysis, forming endings which lie closely apposed to the basal lamina. The basal lamina separates the neurohypophysial neuropil from the perivascular space surrounding each capillary vessel (Figure 2). Since the blood brain barrier is absent in the neurohypophysis, bidirectional movement of substances between the neurohypophysial perivascular space and the systemic circulation is allowed. In addition to allowing secretion of substances from the neurohypophysis into the systemic circulation, this bidirectional movement may allow substances circulating in the blood to affect components of the neurohypophysis such as the pituicytes, which cover much of the basal lamina when the HNS is not activated.

Two peptide hormones, oxytocin and vasopressin, are synthesized in cell bodies of magnocellular neuroendocrine cells and are transported to the neurohypophysis for secretion into the systemic circulation (Buijs *et al.*, 1983). Oxytocin and vasopressin have well

Figure 1. Diagram of the magnocellular hypothalamo-neurohypophysial system. Efferents are shown projecting from the supraoptic nucleus (SON) to the neurohypophysis and from the paraventricular nuclei (PVN) to the neurohypophysis, the median eminence, and to an intrahypothalamic area via axon collaterals.

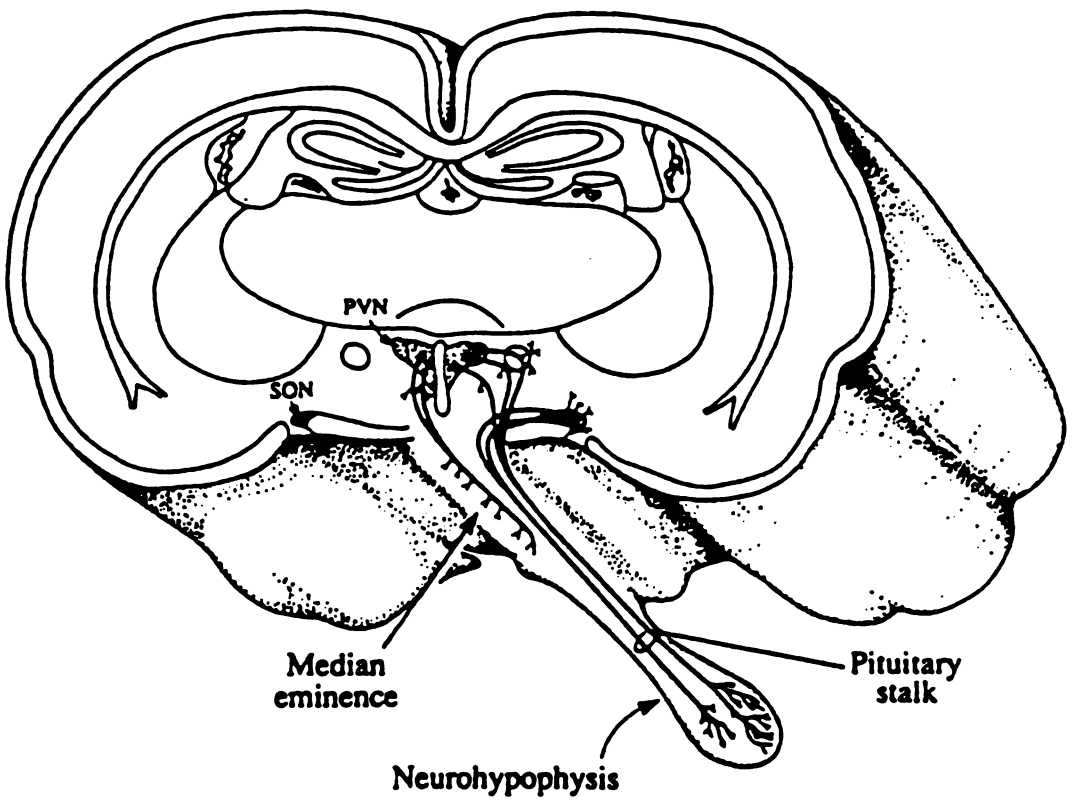


Figure 1

Figure 2. Diagrammatic representation of a neurosecretory area in the rat neurohypophysis. Pituicyte cytoplasm and membrane surrounds neurosecretory axon terminals and is interposed between axon terminals and the basal lamina in unstimulated animals (A). Retraction of pituicyte interpositions from between axon terminals and from between axon terminals and the basal lamina allows increased neurosecretory axon terminal apposition with the basal lamina in animals in which the HNS has been activated (B).

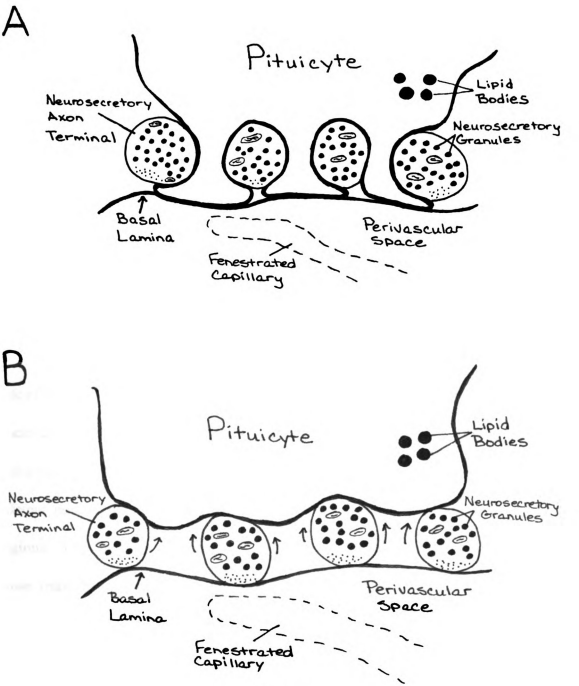

A
Pituicyte
Lipid Bodies
Neurosecretory Axon Terminal
Neurosecretory Granules
Basal Lamina
Perivascular Space
Fenestrated Capillary
B
Pituicyte
Lipid Bodies
Neurosecretory Axon Terminal
Neurosecretory Granules
Basal Lamina
Perivascular Space
Fenestrated Capillary

Figure 2

defined roles in body fluid homeostasis, maintenance of blood pressure, and reproduction. Oxytocin is critically involved in uterine smooth muscle contraction during parturition and in contraction of myoepithelial cells of the mammary gland to produce milk ejection during lactation (Roberts, 1977; Robinson, 1986). Vasopressin is involved in the regulation of renal water absorption (Verney, 1947), and it produces vasoconstriction in certain vascular beds (Pittman *et al.*, 1982). Both oxytocin and vasopressin have been shown, paradoxically, to be natriuretics (Balment *et al.*, 1986).

II. The Paraventricular and Supraoptic Nuclei

The supraoptic and magnocellular paraventricular nuclei are anterior hypothalamic nuclei and are both major components of the HNS. The paraventricular nuclei lie on either side of the dorsal third ventricle and are far from the pial surface of the brain. The supraoptic nuclei are located near the lateral aspects of the optic chiasm and tracts and lie close to the pial surface, but they are several millimeters away from the third ventricle. There are various afferent inputs to the PVN and SON from the forebrain, brain stem, and intrahypothalamic regions. Several of these afferent inputs are common to both the PVN and SON, such as those from the subfornical organ (SFO) (Miselis, 1981) and the ventrolateral medulla (Cunningham and Sawchenko, 1988).

Early lesion studies on magnocellular neurons indicated that vasopressin was of SON origin and that oxytocin originated in the PVN (Nibbolink, 1961; Olivercrona, 1957). However, more recent studies have shown that the PVN and SON each contain populations of oxytocin producing MNCs as well as vasopressin producing MNCs (van Leeuwen and

Swaab, 1977; Vandesande and Dierickx, 1977). Although oxytocin and vasopressin MNCs are found in the SON and PVN, there is a differential distribution of oxytocin MNCs and vasopressin MNCs within the SON and within the PVN. For example, more vasopressin neurons are in the posterior-ventral area of the SON, where as the anterior-dorsal region contains more oxytocin neurons (Swaab *et al*, 1975; Vandesande and Dierickx, 1977).

Hypothalamic magnocellular neuroendocrine cells send out axons which terminate near fenestrated capillaries in the neurohypophysis (Palay, 1957). Approximately 50% of the MNCs that project to the neurohypophysis originate in the SON and magnocellular PVN (Fisher *et al.*, 1979). The remaining MNCs that terminate in the neurohypophysis originate from elsewhere in the anterior hypothalamus (Peterson, 1966) and are an uneven mix of oxytocin- and vasopressin-containing neurons (Rhodes *et al*, 1981). Axons of the MNCs of the SON project through the extreme ventromedial region of the hypothalamus and through the internal zone of the median eminence to the neurohypophysis (Hatton *et al.*, 1984). SON projections terminate selectively in the central part of the neurohypophysis, whereas the PVN magnocellular neurons terminate selectively in the more peripheral regions (Alonso and Asenmacher, 1981).

Magnocellular neuroendocrine cells of the SON and PVN are best known for their projections to the neurohypophysis, although they also send collateral axons from both oxytocin and vasopressin neurons to the lateral hypothalamus (Leng, 1982; Mason *et al.*, 1984; Hatton *et al.*, 1985). There is a significant difference in the number of target areas to which magnocellular neurons of the SON and PVN project, as the distribution of SON efferents is much more conservative than the distribution of PVN efferents. An initial study

by Bodian and Maren (1951) and a variety of anatomical, physiological, and biochemical studies since then have confirmed the connection between the SON and the neurohypophysis. Since retrograde tracers placed into the neurohypophysis back fill almost every SON neuron and electrical stimulation of the neural stalk antidromically activates virtually every SON neuron, it has been determined that the neurohypophysis is the primary target for magnocellular neurons originating from the SON. In contrast, in addition to terminating in the neurohypophysis, PVN magnocellular neurons have been shown to project to the median eminence (Vandesande *et al*, 1977), brainstem and/or spinal cord (Armstrong *et al*, 1980, Hancock, 1976; Hosoya and Matsushita, 1979; Saper *et al.*, 1976; Schwanzel-Fukuda *et al.*, 1984, Swanson, 1977; and Swanson and Kuypers, 1980), and forebrain (Luiten *et al.*, 1985).

III. Magnocellular Neuroendocrine Cells

Magnocellular neuroendocrine cells produce oxytocin and vasopressin in hypothalamic cell bodies in the PVN and SON, and physiological stimuli that activate the HNS evoke increased firing of these neurons and induce secretion of oxytocin and vasopressin from axon terminals in the neurohypophysis. Evidence in the literature indicates that there may even be dendritic release of oxytocin and/or vasopressin in the SON (Hatton, 1990). Magnocellular neurons display electrical activity which can be assigned broadly to three patterns of firing. These include slow irregular, fast continuous, and phasic (Poulain and Wakerley, 1982). The slow irregular pattern has a very low mean firing rate (less than 3 spikes/sec) and is usually considered as the background pattern for unstimulated oxytocin

and vasopressin neurons. Fast continuous electrical activity is unimodal with a mean firing rate of greater than 3 spikes/sec and is observed in both oxytocin and vasopressin neurons. The phasic pattern is bimodal with successive periods of increased neuronal firing and periods of electrical silence occurring in a more or less regular fashion, and it is characteristic of active vasopressin neurons (Wakerley and Lincoln, 1971; Wakerley *et al.*, 1978; Poulain and Wakerley, 1982). Most dye-labeled magnocellular neurons demonstrating phasic activity during intracellular recordings in rat hypothalamic slices are vasopressin-immunoreactive (Yamashita *et al.*, 1983; Cobbett *et al.*, 1986), indicating that phasic activity may be considered a hallmark of vasopressin magnocellular neurons.

Under basal conditions *in vivo* (i.e. animals which are under environmental and physiological conditions that do not cause an increase in the amount of oxytocin or vasopressin released), many neurons of the SON and PVN display slow irregular electrical activity. With activation of the HNS there is a stimulus-evoked increase in oxytocin and/or vasopressin neuronal activity and a corresponding increase in hormone release (see review by Poulain and Theodosis, 1988). For example, suckling during lactation results in fast continuous firing (10-20 Hz) of oxytocin neurons with a characteristic overlying high frequency discharge of action potentials (60-80 Hz) which precedes an increase in intramammary pressure and milk ejection approximately 15 seconds later (Wakerley and Lincoln, 1973). The delay between hormone release and the increase in intramammary presure and milk ejection corresponds to the time necessary for oxytocin to reach and act on the mammary gland (Wakerley and Lincoln, 1973). Oxytocin neurons fire simultaneously to produce a sharply elevated plasma oxytocin concentration and subsequent milk ejection

(Belin and Moos, 1986; Belin *et al.*, 1984; Moos and Richard, 1988, 1989). It is interesting to note that hemorrage and osmotic stimulation (IP injection of hypertonic saline) result in a regular and continuous increase in firing of oxytocin-secreting neurons without the high frequency discharges which are observed during lactation (Poulain and Wakerley, 1982).

Vasopressin neurons can exhibit slow irregular, fast continuous, or phasic electrical activity (see Poulain and Wakerley, 1982). Vasopressin-secreting neurons were first identified in lactating rats from their ability to exhibit a phasic activity in response to conditions which increase the demand for vasopressin release, such as hemorrage (Wakerley *et al.*, 1975), dehydration (Arnauld *et al.*, 1974, 1975), or an elevation in plasma osmotic pressure (Wakerley *et al.*, 1978). In contrast to the brief high frequency bursts of firing that oxytocin-secreting cells exhibit prior to each milk ejection, phasically "bursting" vasopressin-secreting neurons typically display a pattern of consecutive periods of electrical discharge at 7-12 Hz lasting tens of seconds followed by a period of silence for a similar period of time (see Renaud and Bourque, 1991). Interestingly, continuous stimulation at a constant rate of 13 Hz produces a fatigue-like reduction in the release of vasopressin which is quite significant after only 4 minutes (Bicknell *et al.*, 1984). Oxytocin neurons are more resistant to this fatigue at constant rates of 10-13 Hz, and they actually continue to increase the amount of hormone released when driven at rates of 50 Hz or more. This difference in hormone output indicates that there is a difference in the amount of hormone which is required when oxytocin or vasopressin neurons are activated.

Certain physiological stimuli can induce the release of both oxytocin and vasopressin, such as hypovolemia and hemorrhage, hyperosmolality, and circulating angiotensin II. In

the rat, suckling, gastric distension, and systemic administration of cholecystokinin, lithium chloride or copper sulfate induce a selective release of oxytocin, whereas stimulation of renal nerve afferents, carotid occlusion and chemoreceptor activation, and nociceptive somatic stimuli induce the release of vasopressin selectively (see review by Renaud and Borque, 1991). A number of afferent projections innervate the magnocellular nuclei and release neurotransmitters and neuropeptides that have been demonstrated or implicated to regulate the release of oxytocin and/or vasopressin (see reviews by Poulain and Wakerley, 1982; Sladek, 1983; Hatton, 1990; Renaud and Borque, 1991). Biogenic amines which may regulate oxytocin and/or vasopressin release include acetylcholine, norepinephrine, dopamine, histamine, and serotonin. Excitatory amino acids such as glutamate and inhibitory amino acids like GABA may regulate hormone release. And peptides which may modulate oxytocin or vasopressin release include angiotensin II, somatostatin, neuropeptide Y, cholecystokinin, substance P, galanin, atriopeptins, inhibin, opioid peptides, and even oxytocin and vasopressin themselves. Oxytocin has been shown to facilitate its own release (Freund-Mercier and Richard, 1984; Moos and Richard, 1989), and it has been suggested that vasopressin may regulate magnocellular hormone release in the HNS, although a modulatory role for vasopressin in the HNS has not been well defined. Studies in the rat SON (Yamashita *et al.*, 1987) and PVN (Inenaga and Yamashita, 1986) indicate a possible excitatory effect of vasopressin. Others studies have implicated vasopressin in the antidromic inhibition of magnocellular neurons (Nicoll and Barker, 1971), although antidromic inhibition has been demonstrated in the neurons of the genetically mutated and chronically osmotically stressed homozygous Brattleboro rats which cannot synthesize

vasopressin (Valtin *et al.*, 1965), therefore indicating that vasopressin is not the neurotransmitter involved in this type of inhibition in the HNS (Dreifuss *et al.*, 1974; Dyball, 1974).

IV. The Neurohypophysis

The neurohypophysis contains pituicytes (astroglial cells), oxytocin and vasopressin magnocellular neurosecretory axons and terminals, as well as non-neurosecretory axons (Baumgarten *et al.*, 1972). It is densely vascularized with fenestrated capillaries, which are characterized by openings in the walls of their endothelial cells, and is therefore outside the blood-brain barrier. Magnocellular neurosecretory axons enter the neurohypophysis and have endings which terminate near the basal lamina, which is a sheet-like extracellular structure that separates the perivascular space from the neuropil. Under basal conditions, the neurosecretory axons and terminals are filled with dense core vesicles containing peptides which have been synthesized in the magnocellular cell bodies. An MNC axon may contain oxytocin or vasopressin, as well as other peptides such as dynorphin (Watson *et al.*, 1982; Weber *et al.*, 1982; Whitnall *et al.*, 1983), met- or leu- enkephalin, cholecystokinin, substance P, vasoactive intestinal polypeptide, somatostatin, galanin (see review by Hatton, 1990), neurotensin (Kahn *et al.*, 1980), endothelin (Yoshizawa *et al.*, 1990), and nitric oxide synthase (Bredt *et al.*, 1990; Huang *et al.*, 1993; Dawson and Snyder, 1994). Studies have demonstrated that dynorphin (Watson *et al.*, 1982) and cholecystokinin (Martin *et al.*, 1983) colocalize with vasopressin and oxytocin respectively, and there is growing evidence that peptides such as these are involved in regulating hormone secretion from the magnocellular

neuroendocrine cells.

Exocytosis of neurosecretory vesicles can occur from axon terminals near the basal lamina, from axon swellings not in contact with the basal lamina (Morris and Pow, 1988; Tweedle *et al.*, 1988b), and even from unspecialized swellings in the median eminence (Buma and Nieuwenhuys, 1987). The dual criteria of terminal contact with the basal lamina and the presence of microvesicles was used to define neurosecretory terminals in the rat neurohypophysis (Nordmann, 1977). Morphometrically derived estimates indicated that there were approximately 40 million hormone release sites. Since approximately 18,000 neurosecretory neurons in the hypothalamus project to the neurohypophysis, this meant that each neurosecretory axon branched into about 2,000 endings. This high degree of axonal branching was not consistent with earlier light microscopic examination of the neural lobe (Bodian, 1951). A more recent study has helped to explain the discrepancy between the calculated number of terminals in the neurohypophysis and what is actually observed with light microscopic examination by demonstrating that microvesicle-containing neurosecretory terminals make *en passant* contacts with the basal lamina which are often separated from each other by pituicytes (Tweedle *et al.*, 1989a).

Studies investigating catecholaminergic innervation of the neurohypophysis have shown direct projections to the neural lobe from noradrenergic neurons in the A2 region of the brainstem (Garten *et al.*, 1989) and from noradrenergic neurons in the superior cervical ganglion (Saavedra, 1985). It should be noted that lesions of the ventral noradrenergic tract greatly deplete the neurohypophysis of norepinephrine, whereas bilateral cervical ganglionectomy only reduces norepinephrine by 40% in the neurohypophysis (Saavedra,

1985). The significance of noradrenergic innervation of the neurohypophysis will be discussed in a subsequent section. Although the secretory activity of both dopaminergic and noradrenergic neurons in the neurohypophysis is increased with dehydration via prolonged water deprivation or by salt loading from 2% NaCl drinking (Alper *et al.*, 1980, 1982; Holzbauer *et al.*, 1980), dopamine has received more attention, partially because neurohypophysial dopamine concentrations have been found most frequently to be 4 to 10 times greater than those of norepinephrine (Holzbauer *et al.*, 1980). It has been suggested that dopamine may serve a negative feedback function since it has been reported to inhibit both oxytocin (Barnes and Dyball, 1982) and vasopressin (Lightman *et al.*, 1982) release from the neural lobe *in vitro*.

V. Nitric Oxide and Atrial Natriuretic Peptide in the HNS

Nitric oxide is a biological molecule which has important regulatory functions in the cardiovascular, immune, and nervous systems. It has been shown to activate soluble guanylate cyclase and increase cGMP in the brain (Katsuki *et al*, 1977; Miki *et al.*, 1977). Although cGMP can activate cGMP-dependent serine/threonine protein kinase, cGMP-dependent cation channels (Light *et al.*, 1989; Fesenko *et al.*, 1991), and cGMP-dependent phosphodiesterases (Beavo and Reifsnyder, 1990), the functions of cGMP in the brain are essentially unknown.

In vivo, nitric oxide is synthesized by nitric oxide synthase (NOS), which is an enzyme that catalyzes the oxidation of one of the terminal guanidino nitrogens of L-arginine to yield nitric oxide and L-citrulline (Palmer *et al.*, 1988). NADPH (β-Nicotinamide

adenine dinucleotide phosphate, reduced) diaphorase staining is indicative of nitric oxide synthase localization (Dawson *et al.*, 1991), and NADPH-diaphorase may be a form of NOS itself (Hope *et al.*, 1991). Staining for NOS and NADPH-diaphorase has been demonstrated in cell bodies in the supraoptic and magnocellular paraventricular nuclei (Bredt *et al.*, 1990; Vincent and Kimura, 1992), and Huang *et al.* (1993) have demonstrated NOS containing neurons projecting from the paraventricular and supraoptic nuclei to the neurohypophysis. The neurohypophysis, in contrast to the intermediate and anterior lobes of the pituitary, stains intensely for NAPDH-diaphorase (Sagar and Ferriero, 1987) and NOS (Bredt *et al.*, 1990), with NOS being concentrated in the magnocellular neurons and terminals which innervate the neurohypophysis (Bredt *et al.*, 1990; Huang *et al.*, 1993, see Dawson and Snyder 1994).

Several studies indicate a potential regulatory role for nitric oxide in the HNS. Several stimuli which activate the HNS are associated with a change in NOS/NADPH-diaphorase staining and/or with a change in NOS mRNA. NADPH-diaphorase/NOS staining increases in the SON (Pow, 1992; Villar *et al.*, 1994; Calka *et al*, 1994), PVN (Villar *et al.*, 1994), and neurohypophysis (Sagar and Ferriero, 1987) after dehydration and salt loading. NOS mRNA is increased in the PVN in young and old rats after immobilization stress (Calzà *et al.*, 1993), in the PVN of lactating rats (Ceccatelli and Eriksson, 1993), and in the PVN and SON of rats after salt loading (2% NaCl in drinking water) (Villar *et al.*, 1994).

One study has demonstrated an enhanced rise in plasma levels of oxytocin, but not vasopressin, in conscious rats following 24 hr water deprivation with treatment of the NOS inhibitors NMMA and L-NAME *in vivo* (Summy-Long *et al.*, 1993), suggesting that nitric

oxide in the HNS attenuates oxytocin release selectively during dehydration. Double labeling experiments have demonstrated colocalization of vasopressin and NOS/NADPH-diaphorase in the PVN and SON (Calka and Block, 1993; Villar *et al.*, 1994). However, Villar *et al.* (1994) noted no preferential distribution of NOS-immunoreactive cells, suggesting that nitric oxide is produced in both vasopressin and oxytocin containing neurons. These studies indicate a potential role for nitric oxide and therefore cGMP to influence magnocellular neuroendocrine cells and therefore regulate hormone secretion from the neurohypophysis into the systemic circulation.

Atrial natriuretic peptide (ANP) is a biologically active peptide involved in renal, adrenal, and cardiovascular regulation. In the brain, the majority of the ANP-responsive, cGMP producing cells have been shown to be astrocytes (see deVente and Steinbusch, 1992), and ANP-immunoreactivity has been demonstrated in a small percentage of magnocellular oxytocin neurons (Jirikowski *et al*, 1986). The effects of ANP on vasopressin secretion from the neurohypophysis is controversial. ANP has been reported to both suppress (Montes and Johnson, 1990) and enhance (Januszewicz *et al.*, 1995) vasopressin secretion *in vivo*. Nissen *et al.* (1989) have indicated that ANP released from perfused basal forebrain explants originates from the median eminence and neurohypophysis. Endogenous ANP and ANP receptors have been demonstrated in the neurohypophysis (Gutkowska *et al.*, 1987), and ANP receptors are localized on cultured pituicytes (Luckman and Bicknell, 1991). Although Luckman and Bicknell showed no effect of ANP on basal or electrically stimulated release of oxytocin or vasopressin from isolated neural lobes or neurointermediate lobes *in vitro* (Luckman and Bicknell, 1991), other investigators have demonstrated that ANP reduces

stimulated release of neurohypophysial hormones *in vitro* from hypothalamo-neurohypophysial explants (Crandall and Gregg, 1986) and from isolated neurointermediate lobes (Poole *et al.*, 1987). ANP receptors have an intrinsic particulate guanylate cyclase, which is an enzyme that produces cGMP and is localized in both pituicytes and in neurosecretory terminals in the neurohypophysis (Rambotti *et al.*, 1994). These studies indicate a potential role for ANP and therefore cGMP to regulate hormone secretion or to modulate pituicyte structure or function. It is unknown whether there are neurotransmitters other than ANP and NO which may alter intracellular cGMP concentration in the neurohypophysis.

VI. Plasticity in the HNS

A number of studies over the last twenty years have provided evidence for structural as well as functional plasticity in the HNS of adult animals in response to certain physiological conditions which activate the hypothalamo-neurohypophysial system. Ultrastructural changes occur in both the hypothalamus and in the neurohypophysis in response to activation of the HNS (Hatton *et al.*, 1984). Under basal conditions (i.e. when there is little demand for oxytocin or vasopressin release) hypothalamic astroglial cells are interposed between magnocellular cell bodies and dendrites in the SON and PVN, and astroglial cells (pituicytes) interpose cytoplasmic and membranous material between magnocellular neurosecretory axons and between the axon terminals and the basal lamina in the neurohypophysis.

Activation of the HNS occurs in response to stimuli such as dehydration, parturition,

and lactation and is associated with dramatic ultrastructural plasticity between the MNCs and astroglia in the SON (Tweedle and Hatton , 1976, 1977; Hatton and Tweedle, 1980, 1982; Theodosis *et al.*, 1981) and PVN (Gregory *et al.*, 1980; Theodosis and Poulain, 1989) and between the MNCs and the pituicytes in the neurohypophysis (Tweedle and Hatton 80a, 1982, 1987). Increased demand for oxytocin or vasopressin release occurs in conjunction with: 1) changes in the hypothalamus, such that magnocellular cell bodies and/or their dendrites may become directly apposed, and new synapses can form, which allows individual axon terminals to make contact with two or more cell bodies or dendrites, and 2) changes in the neurohypophysis, whereby there is an increase in neuronal apposition with the basal lamina and a corresponding decrease in the amount of coverage of the basal lamina by pituicytes (see review by Hatton, 1990).

A. Plasticity in the Hypothalamus

When the hypothalamo-neurohypophysial system is activated, there are dramatic changes in the ultrastructural organization of the hypothalamic magnocellular nuclei where the cell bodies of MNCs are located, as they undergo alterations in electrical, biosynthetic, and secretory activities (Theodosis and Poulain, 1989; Theodosis *et al.*, 1981; and Tweedle and Hatton, 1984). The ultrastructural changes resulting from activation of the HNS have been best characterized in the SON (Gregory *et al.* 1980; Hatton, 1985; Hatton and Walters, 1973; Modney and Hatton, 1989a; Tweedle and Hatton, 1977 and 1984), although ultrastrastructural alterations have also been shown to occur in the PVN when the HNS is stimulated (Gregory *et al.*, 1980; Theodosis and Poulain, 1989).

Both subcellular and intercellular changes have been shown to occur with activation of the HNS. One subcellular change which occurs in the hypothalamic magnocellular neuroendocrine cells in response to activation of the HNS by dehydration from 24 hour water deprivation is an increase in the number of nucleoli within the nucleus of each oxytocin and vasopressin neuron in the SON (Hatton and Walters, 1973) and PVN (Hoblitzell *et al.*, 1976). Additional subcellular changes in MNCs which occur in response to dehydration from water deprivation or from drinking 2% NaCl include increased levels of ribosomal RNA (Kawata *et al.*, 1988) and increased amounts of mRNA for vasopressin, dynorphin (Sherman *et al.*, 1986a and b, 1988), tyrosine hydroxylase (Young III *et al.*, 1987b, Watts 1992), galanin (Meister *et al.*, 1990; Young III *et al.*, 1990), cholecystokinin (Sherman *et al.*, 1988), vasoactive intestinal peptide (Watts, 1992), neuropeptide Y (Larsen *et al.*, 1992a and b), nitric oxide synthase (Calzá *et al.*, 1993; Ceccatelli and Eriksson, 1993) and G-proteins (Young III *et al.*, 1987a).

Several forms of intercellular plasticity have been observed between hypothalamic elements of the HNS. Eneström (1967) first demonstrated direct membrane appositions between SON cell bodies in the hypothalamus of rats. Membrane appositions between magnocellular endocrine cell bodies and/or magnocellular neuroendocrine dendrites increase when the HNS is activated, and membrane appositions have been shown to vary as a direct function of an animal's hydration state (Tweedle and Hatton, 1976, 1977). Retraction of astroglial cells from between SON cells and increased direct soma-somatic appositions in the SON occurs when an animal is dehydrated by drinking 2% NaCl solution (Lafarga *et al.*, 1975; Tweedle and Hatton, 1983) or by water deprivation (Tweedle and Hatton 1976, 1977;

Gregory *et al.*, 1980). These ultrastructural changes in the SON which occur from water deprivation (Tweedle and Hatton, 1977) or from drinking a 2% NaCl solution (Tweedle and Hatton, 1983, 1984) have been shown to be reversible when the animal is rehydrated. Beagley and Hatton have demonstrated that an intraperitoneal injection of 1.5 M NaCl will not only increase soma-somatic appositions in the SON (1992), but will also induce a significant decrease in glial apposition and a concomitant increase in somatic and afferent axon terminal apposition with the MNC membrane in the SON compared to control animals (1992, 1994). Gregory *et al.* (1980) have demonstrated soma-somatic appositions between magnocellular neuroendocrine cells in the PVN in response to dehydration.

Lactation and parturition, which are physiological stimuli causing activation of the HNS and increased oxytocin release, are also associated with a marked decrease in glial coverage of MNC somata and dendrites in the SON and PVN and a large increase in neuronal juxtapositions (Tweedle and Hatton, 1976). Soma-somatic appositions between magnocellular neuroendocrine cells in the SON have been shown to occur to a much greater extent during lactation as compared to during dehydration (Hatton and Tweedle, 1980, 1982; Theodosis *et al.*, 1981). It has also been reported that lactating rats have significantly more oxytocin soma-somatic appositions than vasopressin soma-somatic appositions in the PVN (Theodosis and Poulain, 1989) and that osmotic stimulation (Chapman *et al.*, 1986) and lactation (Theodosis *et al.*, 1986a) are associated with ultrastructural alterations within the SON of the rat which affect oxytocinergic but not vasopressinergic cells. As the extent and occurrence of direct neuronal surface membrane appositions between neurosecretory elements in the SON increase dramatically during lactation (Theodosis *et al.*, 1981), it has

been suggested that these extensive neuronal appositions which result from a lack of interposing glia may lead to electrical interactions between the adjacent neurons which can facilitate synchronization of neuronal firing.

When the HNS is not activated, dendrites of magnocellular neuroendocrine cells have interposing astroglial cells between them. Dendrites of magnocellular neurons also form close appositions and form bundles of dendrites with no interposing astroglial cells separating them. Dendritic bundling occurs with dehydration (Perlmutter *et al.*, 1985), during late gestation (Perlmutter *et al.*, 1984b; Taubitz *et al.*, 1987; Theodosis and Poulain, 1984), and in maternally behaving virgin rats (Salm *et al.*, 1988). Dendritic bundling is reversible upon removal of the stimulus which is activating the HNS and when there is a return to conditions which do not require elevated hormone output (Perlmutter *et al.*, 1985). It has been suggested that dendritic bundling due to removal of the interposing astroglia from between dendrites may serve to increase the excitability of the HNS neurons by synchronizing groups of neurons and/or by removing inhibitory or buffering effects which may occur via the interposing astroglial cells (Hatton, 1990).

"Normal" synapses are defined as one nerve terminal contacting one post-synaptic element, such as a cell body. Multiple synapses are rarely observed in animals in which the HNS is not activated. Multiple synapses are single synaptic terminals making contact with more than one postsynaptic element, and they occur in the SON and PVN when the HNS is activated (Lafarga *et al.*, 1975). The number of multiple synapses is increased in the somatic areas of the SON (Theodosis *et al.*, 1981, Tweedle and Hatton, 1982) and PVN (Theodosis and Poulain, 1989) of lactating animals compared to untreated virgin rats as well as in the

SON dendritic zone during parturition (Perlmutter *et al.*, 1984b). Male and female rats which have become dehydrated by drinking hypertonic saline water and chronically dehydrated, vasopressin deficient homozygous Brattleboro rats have increased numbers of multiple synapses in the SON somatic region (Chapman *et al.*, 1986; Modney and Hatton 1989a, Tweedle and Hatton 1984). In "normal" rats which are not vasopressin deficient, the number of multiple synapses observed under the condition of increased hormone demand returns to normal in the absence of stimuli which activated the HNS (Tweedle and Hatton, 1984).

The formation of multiple synapses was proposed to occur from retraction of astroglia from between neurons and axon terminals (Hatton and Tweedle, 1982; Hatton *et al.*, 1988). The paradoxical evidence that chronically dehydrated homozygous Brattleboro rats have increased somatic appositions and increased glial coverage of SON neurons (Chapman *et al.*, 1986) initially disputed the proposed mechanism by Hatton and colleagues, but a subsequent study by Modney and Hatton (1989a) provided supporting evidence in favor of the initial proposed mechanism. They demonstrated that when genetically intact rats, which are able to synthesize vasopressin, are given 2% saline to drink for 10 days that glial retraction is an early response which allows increased membrane appositions and new synapses to form; then as stimulation becomes chronic, glial coverage actually increases to maintain a constant proportion of the membrane of the enlarging secretory cell in contact with the astroglia.

Whether a stimulus is acute or chronic appears to be associated with somewhat different effects on magnocellular neuro-glial plasticity. In the hypothalamus, acute

stimulation of the HNS is associated with glial retraction from between dendrites and from between cell bodies of SON neurons. Long term elevation of hormone demand may induce glial proliferation (Paterson and Leblond, 1977; Murray 1968) and induce the formation of new synapses (see Hatton, 1990). In addition, although both acute and chronic dehydration induces dendritic bundling of SON dendrites (Perlmutter *et al.*, 1985), effects of acute dehydration can be reversed by a period of rehydration equal to the dehydration period, but effects of chronic dehydration take a much longer period of rehydration to reverse. This may indicate that the HNS adapts and makes more permanent functional or structural alterations with chronic stimulation as opposed to more temporary modifications with acute stimulation of the HNS. Also, the area of SON magnocellular neuronal cell bodies increases and the terminals contacting SON cell bodies enlarge in response to prolonged dehydration by drinking 2% NaCl for example (Modney and Hatton, 1989a). Modney and Hatton (1989a) have proposed that the increase in terminal size occurs to maintain a constant synaptic density on larger MNC cell bodies.

There are several lines of evidence indicating that glial retraction occurs by active withdrawal of the astroglial cells as opposed to enlarging neurons pushing the astroglial cells out of the way. Ultrastructural changes in the HNS are established by 4 hr of water deprivation and are significant by 24 hr of water deprivation (Tweedle and Hatton, 1977). Since ultrastructural changes in the SON occur much earlier than increases in the size of the MNCs in the SON, which requires a minimum of 24 hours to be measured at the light microscopic level (Hatton and Walters, 1973), it is likely that the glia actively withdrawal their cytoplasm from between the MNCs rather than being pushed out by neuronal swelling.

Dendritic bundling, which requires the removal of the interposing astroglial cells also occurs with no increase in the size of dendrites. Lastly, new or multiple synapse formation occurs in minutes to hours (Modney and Hatton 1989b, Tweedle *et al.* 1989b) which is consistent with rapid glial retraction, but not with an increase in the size of SON or PVN cell bodies.

B. Plasticity in the Neurohypophysis

Electron microscopy has demonstrated a close association between pituicytes, which are GFAP (glial fibrillary acidic protein) positive astroglia (Suess and Pliška, 1981) and the predominant cell type of the neurohypophysis, and the neurohypophysial neurosecretory axons and axon terminals (Palay, 1957). Under basal conditions, pituicytes surround and engulf MNC axons and axon terminals in the neurohypophysis (Figure 2). Pituicyte cytoplasmic and membranous material is also interposed between the axon terminals and the basal lamina to form a physical barrier that may reduce access of hormones secreted from the terminals into the systemic circulation. It has been suggested that pituicytes may also reduce hormone secretion by influencing extracellular ion concentrations of axon terminals surrounded by pituicytes or by releasing substances onto neurosecretory axons or axon terminals to cause receptor mediated inhibition of hormone secretion (Tweedle and Hatton, 1982).

Studies have shown that dynamic interactions occur between the neurosecretory axon terminals and pituicytes (Tweedle and Hatton, 1980a, 1980b, 1982, 1987). Increased hormone secretion during parturition, lactation, or dehydration is associated with ultrastructural changes in the neurohypophysis. *In vivo*, stimuli which increase oxytocin

and/or vasopressin release also induce a decrease in the number of neurosecretory axons enclosed by pituicytes (Tweedle and Hatton, 1980a and b, 1982) (Figure 2). There is also an increase in the amount of neurosecretory terminal apposition and a corresponding decrease in astroglial contact with the basal lamina (Tweedle and Hatton, 1980a and b; Wittkowski and Brinkmann, 1974) (Figure 2). Activation of the HNS appears to cause pituicytes to change ultrastructurally and retract their cytoplasmic material from between axon terminals and from between axon terminals and the basal lamina. Diminished stimulus-evoked hormone secretion, for example with rehydration, is associated with reinsertion of pituicytes between axon terminals and between axon terminals and the basal lamina, such that the number of axon terminals enclosed by pituicytes increases and returns to basal levels (Tweedle and Hatton, 1980a). Hormone secretion into the systemic circulation may therefore be facilitated by removal of the pituicyte-formed physical barrier which is observed under basal conditions, and it is likely that pituicyte plasticity in general plays an instrumental role in regulating the release of oxytocin and vasopressin into the systemic circulation. It is most likely that the physiological sequence of events which occurs when the HNS is activated is the following: 1) The neurons within the HNS are activated by dehydration, lactation, etc.; 2) Release of oxytocin and vasopressin and other neurohypophysial hormones and neurotransmitters from the axon terminals of magnocellular neurons is increased; 3) Oxytocin and vasopressin enter the systemic circulation; 4) Ultrastructural changes in the HNS (hypothalamus and neurohypophysis) are initiated; 5) Ultrastructural changes modulate subsequent hormone release.

As was observed in the SON, acute and chronic stimulation of the HNS appear to

have differential effects on plasticity in the neurohypophysis. Short term activation of the HNS, such as with acutely elevated levels of testosterone (Tweedle *et al.*, 1988a) or with mild water deprivation during parturition (Tweedle and Hatton, 1987), results in enlarged terminal contacts with the basal lamina. However, chronic activation of the HNS with lactation (Tweedle and Hatton, 1987) or with prolonged elevation of testosterone (Tweedle *et al.*, 1988a) causes an increase in the number of terminals contacting the basal lamina without altering the size of the neurosecretory terminals. There is also a difference in the amount of time it takes to reverse the ultrastructural effects induced by acute vs. chronic stimulation of the HNS. In the case of acute stimulation, a 24 hr rehydration period is sufficient to reverse the effects of 24 hr of dehydration on pituicyte ultrastructure in the neurohypophysis (Tweedle and Hatton, 1980a). However, with chronic stimulation of the HNS from 10 days of drinking 2% NaCl, a complete return to the ultrastructure which is similar to that of an unstimulated animal is not observed until 5 weeks of rehydration (Tweedle and Hatton, 1987).

C. What Induces Neurohypophysial Ultrastructural Changes?

Although it has been established that physiological stimuli which activate the HNS induce ultrastructural changes and glial retraction in the neurohypophysis, the mechanism by which these physiological changes act and the biochemical mechanisms mediating pituicyte plasticity remain an enigma. As discussed previously, oxytocin, vasopressin, dynorphin, endothelin, and a variety of other peptides have been identified in the neurohypophysis. The neural lobe also receives central innervation from fibers containing

norepinephrine, dopamine, serotonin, and gamma amino butyric acid (see Hatton, 1990). Since the neurohypophysis is densely vascularized with fenestrated capillaries, the blood brain barrier is absent in the neurohypophysis, and it is possible that stimuli which activate the HNS and induce ultrastructural changes in the neurohypophysis could also be peripheral in origin. Any one or a combination of these hormones or transmitters could potentially affect neurohypophysial ultrastructure, providing that the pituicytes contain the necessary receptors to respond to a particular stimulus.

Similar to what is observed for other astroglial cells, pituicytes have receptors for blood-borne transmitters and hormones and for neurotransmitters and hormones released within the neurohypophysis from magnocellular neuroendocrine cells such as dynorphin (Bicknell *et al.*, 1989), endothelin (Ritz *et al.*, 1992), atrial natriuretic peptide (Luckman and Bicknell, 1991), and vasopressin (Hatton *et al.*, 1992). Pituicytes also have β_2 adrenoreceptors (Bicknell *et al.*, 1989; Hatton *et al.*, 1991) which may mediate effects in response to norepinephrine released within the neurohypophysis or in response to circulating epinephrine. There have been very few studies to investigate whether particular peptides or neurotransmitters affect pituicyte ultrastructure *in vivo* or *in situ* or affect pituicyte morphology *in vitro*. Although pituicytes have kappa-opioid receptors for dynorphin, dynorphin has not been shown to induce stellation of pituicytes *in vitro* (Bicknell *et al.*, 1989). Endothelin has been localized in isolated neurosecretory nerve endings and has been shown to potentiate vasopressin release from electrically stimulated isolated neural lobes (Ritz *et al.*, 1992). Although endothelin increases intracellular calcium levels in pituitcytes, there is no evidence indicating that endothelin affects pituicyte morphology (Ritz *et al.*,

1992). Interestingly, endothelin has been shown to prevent stellation induced by isoproterenol, forskolin, dibutyryl cAMP, and phorbol-12-myristate-13-acetate (PMA) in rat cerebral cultured astrocytes (Koyama *et al.*, 1993).

Two neuropeptides that would seem like obvious potential modulators for pituicyte morphology are oxytocin and vasopressin. Theodosis *et al.*, (1986b) have demonstrated that intracerebroventricular administration of oxytocin is associated with morphological changes in the SON, but this has not been reported to occur in the neurohypophysis. Vasopressin (V1) receptors are present on pituicytes (Hatton *et al.*,1992), and although vasopressin increases intracellular calcium concentration in pituicytes, it has not been shown to affect pituicyte morphology *in vitro*. However, subcutaneous administration of vasopressin to Brattleboro rats has been shown to significantly decrease the number of appositions per cell body and decrease the total length of membrane apposition per cell body (Chapman *et al.*, 1986). This study indicates that vasopressin affects ultrastructural relations in the SON, although it is unclear whether the effects are direct or indirect.

Nitric oxide synthase and atrial natriuretic peptide have been identified in the neurohypophysis and ANP receptors have been localized to pituicytes. Although there are no previous studies on the effects of ANP on pituicyte morphology, one study has investigated the effects of nitric oxide on ultrastructural relations in the neurohypophysis. Beagley and Cobbett (1996) have demonstrated that compared to rats given an intraperitoneal injection of 1.5 M NaCl alone, rats injected with both 1.5 M NaCl and the nitric oxide synthase inhibitor L-NAME have a significantly smaller amount of axon terminal contact with the basal lamina and a significantly higher number of axon terminals

enclosed by pituicytes in the neurohypophysis. Although this study implicates nitric oxide as a regulator of pituicyte ultrastructure *in vivo*, L-NAME was not administered directly into the neurohypophysis, and therefore its site of action is unclear.

Steroid hormones may also be involved in regulating pituicyte morphology. Estradiol has been shown to promote changes in cell morphology of cultured hypothalamic astrocytes (Torres-Aleman *et al.*, 1992), and if pituicytes have the appropriate receptors, it could potentially modulate pituicyte morphology *in vivo.* Alterations in circulating levels of testosterone have been associated with changes in pituicyte ultrastructure *in vivo.* Castration has been shown to not only to decrease coupling among magnocellular neurons in the PVN (Cobbett *et al.*, 1987), but it actually induces an increase in the amount of neurosecretory terminal enclosure by pituicytes and a decrease in the amount of basal lamina occupied by neurosecretory terminal membrane (Tweedle *et al.*, 1988a). Just as dehydration-induced ultrastructural changes can be reversed with rehydration, the effects of castration on HNS ultrastructure can be reversed by replacement of testosterone (Tweedle *et al.*, 1988a). The fact that castration also decreases circulating levels of vasopressin (Crofton *et al.*, 1985) provides supporting evidence that pituicytes form a physical barrier which functions to reduce hormone entry into the systemic circulation.

Both *in vivo* and *in vitro* experiments have shown that ultrastructural changes in the neurohypophysis can be induced by osmotic manipulation. *In vivo*, water deprivation (Tweedle and Hatton, 1980b), salt loading by administering 2% NaCl in the drinking water (Tweedle and Hatton, 1987), and intraperitoneal hypertonic saline injections (Beagley and Hatton, 1992, 1994) have been shown to induce retraction of pituicyte cytoplasmic material

from the axon terminals and from between axon terminals and the basal lamina. *In vitro* studies have demonstrated that manipulation of the osmotic concentration of the solution in which isolated neurointermediate lobes are incubated also affects pituicyte ultrastructure in a manner which is analagous to ultrastructural changes induced by altering osmotic concentration *in vivo* (Perlmutter *et al.*, 1984a). The number of axons surrounded by pituicytes is inversely related to the osmolarity of the medium, and the amount of neural apposition with the basal lamina is directly related to the osmolarity of the medium (Perlmutter *et al.*, 1984a). Since there is no connection between the neurohypophysis and the hypothalamus in the isolated neurointermediate lobe preparation, it appears that changes in osmolarity may alter pituicyte ultrastructure directly.

Beagley and Hatton (1994) have demonstrated that bilateral adrenal medullectomy can inhibit osmotically induced (by IP injection of 1.5 M NaCl) ultrastructural changes in the neurohypophysis, suggesting that circulating epinephrine, for example, may provide a peripheral signal to stimulate retraction of pituicytes *in vivo*. Although circulating epinephrine may play a critical role in neurohypophysial ultrastructural changes in response to osmotically-induced stimulation of the HNS, there are other factors which likely contribute to or modulate these effects. For example, the subfornical organ has a direct projection to the magnocellular hypothalamic nuclei and is sensitive to changes in blood osmolarity (see review by Dellman, 1985). Signals from the SFO to the SON and PVN may provide a direct central input for mediating ultrastructural changes in the SON and PVN and possibly an indirect central input to the neurohypophysis.

Neurointermediate lobes (a neurohypophysis with the pars intermedia still attached),

which were isolated from adult rats, were perfused with a β-adrenergic agonist-containing solution, and they exhibited retraction of pituicytes from between axon terminals and the basal lamina and had an increase in neural contact with the basal lamina with a correlated decrease in pituicyte coverage of the basal lamina (Smithson *et al*, 1990; Luckman and Bicknell, 1990). These studies suggest that β-adrenoreceptor activation can mediate retraction of pituicytes in the neurohypophysis. It is likely that β-adrenoreceptor activation induces an increase in intracellular cAMP concentration to alter pituicyte ultrastructure. One study has demonstrated that cAMP is increased in neurohypophyses obtained from NaCl-treated and lactating rats (Ruoff *et al.*, 1976). This supports the hypothesis that increased levels of intracellular cAMP can modulate pituicyte ultrastructural changes *in vivo* . Since the MNCs are cut when using the isolated neurointermediate lobe preparation, connections between the neurointermediate lobe and the hypothalamus do not seem to be necessary for these ultrastructural changes to occur, and the isolated neurointermediate lobe appears to be a viable model to represent morphological plasticity which occurs in the neurohypophysis *in vivo*.

Although the neurohypophysis was known to contain β_2 adrenergic receptors (De Souza, 1985) and retraction of pituicyte material could occur in neurointermediate lobes in response to β-adrenergic and osmotic stimuli, it was still unclear whether these ultrastructural changes were mediated by receptors on pituicytes. Bicknell *et al.* (1989) developed a method to culture pituicytes from adult rats to investigate if receptors on pituicytes could mediate ultrastructural changes in the absence of neurosecretory neurons. Pituicytes cultured from adult rat neurohypophyses were shown to change morphologically

from a flat amorphous shape, with no distinct cytoplasmic processes and no distinct cell body (non-stellate), to a stellate form, which has cytoplasmic processes and a distinct cell body, in response to β-adrenoreceptor activation (Bicknell *et al.*, 1989). Noradrenergic-induced changes in pituicyte morphology have been demonstrated to be mediated by β_2-adrenergic receptors, since the β_2-adrenergic antagonist IPS 339 blocked norepinephrine-induced stellation of cultured pituicytes, but practolol (Hatton *et al.*, 1991), prazosin, and yohimbine (Bicknell *et al.*, 1989), which are β_1, α_1, and α_2 adrenergic antagonists respectively did not.

Beagley and Hatton (1994) have demonstrated that circulating epinephrine may be involved in regulating pituicyte ultrastructure *in vivo*. It is possible that circulating epinephrine acts on pituicyte β-adrenoreceptors to exert its effects. In addition, norepinephrine remains a possible source of stimulation to induce ultrastructual plasticity of pituicytes via β-adrenoreceptors. Three possible sources for norepinephrine to affect pituicyte ultrastructure *in vivo* include: 1) CNS noradrenergic innervation from the A2 cell group of the mediodorsal medulla oblongata, 2) sympathetic innervation from the superior cervical ganglia, and 3) circulating catecholamines from the adrenal medulla.

VII. Astroglial Plasticity

Morphological plasticity has been demonstrated in astroglial cell line cultures and in astroglial cells derived from a number of brain regions. Several studies have demonstrated that β-adrenergic agonists, direct activators of adenylate cyclase, and cAMP analogues can cause astrocytes in primary cell culture (Narumi *et al.*, 1978; Tardy *et al.*, 1981; Federoff *et al.*, 1984; Pollenz and McCarthy, 1986) and astroglial cells from cell lines (Oey 1975; Shain

et al., 1987) to change morphologically from a flat, epithelial-like form to a stellate, process-bearing morphology. As there are numerous studies indicating that elevated intracellular cAMP concentration will induce stellation in astrocytes, it has been proposed that changes in astroglial morphology result from activation of cAMP-dependent kinase (PKA) (Browning and Ruina, 1984; McCarthy *et al.*, 1985). Unfortunately there is a paucity of studies describing a biochemical pathway which links PKA activation and cytoskeletal changes.

Recent studies have demonstrated that agents other than those which elevate intracellular cAMP concentration can also modulate astroglial morphology. ATP (Neary *et al.*, 1994) and vasoactive intestinal peptide (VIP) (Hisanaga *et al.*, 1993) have been shown to induce stellation of primary cortical astrocyte cultures, although the biochemical mechanisms mediating ATP- and VIP-induced stellation is not known. In contrast to agents which induce stellation, glutamate, endothelin, serum, and lysophosphatidic acid (LPA), a component of serum, have been shown to prevent and reverse stellation in cultured astrocytes. Glutamate can inhibit isoproterenol, dBcAMP, and phorbol-12-myristate-13-acetate (PMA) induced stellation of astrocytes cultured from neoneatal rat cerebral cortex (Shao *et al.*, 1994). Although glutamate was shown to reduce cAMP production by 25%, norepinephrine, which induces stellation, actually reduced cAMP formation by over 80%, indicating that the reduction of cAMP cannot account for the effects of glutamate on stellation (Shao *et al.*, 1994). Since glutamate prevents both cAMP- and PMA-induced stellation, it has been suggested that glutamate acts through a mechanism which is common to both PKA and PKC pathways involved in affecting cell morphology (Shao *et al.*, 1994).

Endothelin has been shown to prevent stellation induced by isoproterenol, forskolin,

dBcAMP, and PMA in cultured rat cerebral astrocytes (Koyama *et al.*, 1993). This indicates that endothelin, like glutamate, may act through a mechanism common to PKA and PKC to affect cell morphology. It has been suggested that endothelin prevents stellation via a cAMP- and calcium-independent mechanism, since it prevents dBcAMP-induced stellation without altering cAMP accumulation and in the absence of calcium (Koyama *et al.*, 1993). In addition, endothelin prevents and reverses cytochalasin-B-induced stellation and reorganizes actin stress fibers which have been shown to disappear with dBcAMP and cytochalasin-B mediated stellation (Koyama and Baba, 1994). This may indicate that endothelin has more of a direct effect on the actin cytoskeleton.

Serum and LPA have been shown to affect astrocyte morphology *in vitro*. β-agonist induced stellation of C6 glioma cells can be reversed by serum and LPA (Koschel and Tas, 1993; Nelson and Simon, 1990; Cavanaugh *et al.*, 1990). Investigators have observed that agents which induce stellation are less effective in inducing stellation when serum is present in the experimental medium (Moonen *et al.*, 1975; Kimelberg *et al.*, 1978). Since the neurohypophysis is outside the blood brain barrier, pituicytes are exposed to serum and there is the potential for serum to affect pituicyte ultrastructure *in vivo*.

Primary astrocyte cultures and glial cell lines exhibit morphological plasticity *in vitro*. The functional significance of astroglial plasticity *in vivo* likely depends on where the cells are located in the brain and the extracellular mileu to which they are exposed. For example, astroglial plasticity in the hippocampus may contribute to memory function, whereas plasticity of pituicytes in the neurohypophysis may regulate hormone release. It is now clear that astrocytes are not simply "nerve glue" as indicated by the German pathologist

Rudolf Virchow who coined the term "neuroglia" in 1846. Astrocytes play key roles in brain development, normal physiology, and pathology of the nervous system, and astroglial plasticity is proving to be an integral part of brain function.

VIII. Alterations in the Cytoskeleton During Morphological Changes

The cytoskeleton is a complex interconnecting network of microfilaments, microtubules, and intermediate filaments in cells and is involved in regulating cell shape, contractility, metabolism, and locomotion. cAMP-induced stellation of cultured astrocytes (Moonen *et al.*, 1976; Goldman and Chiu, 1984; Federoff *et al.*, 1987) has been described as a peripheral cytoplasmic contraction which leaves behind branched processes that emanate from a reduced cell body (Goldman and Abramson, 1990). Several lines of evidence indicate that cytoskeletal proteins such as microtubules, actin, and intermediate filaments are involved in the alteration of cell morphology.

Dibutyryl-cAMP-induced stellation of astrocytes occurs by tubulin-dependent changes in the organization of the cytoskeleton (Goetschy *et al.*, 1986; Goldman and Abramson, 1990). In astrocytes, a substantial portion of tubulin is assembled into microtubules (Goetschy *et al.*, 1986). Colchicine is an agent which depolymerizes microtubules, and thus makes the cytoskeletal system somewhat disfunctional. Colchicine can prevent cAMP-dependent stellation of cells from astroglial cell lines (Shain *et al.*, 1992) and stellation of cultured cortical astrocytes (Goetschy *et al.*, 1986; Goldman and Abramson, 1990). Taxol, a microtubule stabilizing agent, has been shown to inhibit the effects of colchicine on dB-cAMP induced stellation (Goldman and Abramson, 1990), further

indicating that a functional microtubule system is essential for shape changes to occur.

Rearrangement of actin, another major cytoskeletal component, appears to be modulated by agents which alter intracellular cAMP concentration, since treatment of cultured astrocytes with dB-cAMP has been shown to significantly reduce the amount of cytoskeletal-associated actin (Goldman and Chiu, 1984). Direct injection of the PKA catalytic subunit results in the loss of actin bundles and rounding up of fibroblasts with concomitant phosphorylation of myosin light chains (Lamb *et al.*, 1988). This suggests that PKA regulates microfilaments through myosin light-chain kinase in these cells (Goldman and Abramson, 1990). Disruption of actin filaments with cytochalasin-B, an inhibitor of actin polymerization, induces stellation (Barodo *et al.*, 1992; Koyama and Baba, 1994) and the disappearance of cables of actin, known as stress fibers, in astrocytes (Koyama and Baba, 1994). This is consistent with the finding that there is a rapid shift of insoluble actin to soluble actin, and therefore collapse of the actin cytoskeleton, associated with dB-cAMP-induced stellation (Goldman and Abramson, 1990). Since endothelins prevent and reverse cytochalasin-B and cAMP-induced stellation and stimulate reorganization of actin stress fibers (Koyama and Baba, 1994), it has been suggested that endothelins function as extracellular signals to regulate cytoskeletal actin organization of astrocytes. It is interesting to note that both LPA (Moolenaar et al., 1992) and endothelin (Krisch and Mentlein, 1994) induce phosphatidylinositol hydrolysis and stimulate an increase in intracellular calcium concentration.

A. Filamentous Cytoskeletal Proteins

Glial fibrillary acidic protein (GFAP) and vimentin are two intermediate filament (IF) proteins frequently used in identification of rodent astrocytes growing in culture (see Goetschy *et al.*, 1986). The biological functions of GFAP and vimentin in atrocytes are not fully understood, but the loss of solubility of GFAP and vimentin and high accumulation of intermediate filament proteins in dB-cAMP-induced stellate astrocytes (Ciesielski-Treska *et al.*, 1984; Chiu and Goldman, 1984) indicate that they play a structural role in astrocytes. Although several studies have indicated that alterations in levels and phosphorylation status of GFAP and vimentin may correlate with particular morphological states of astroglial cells, the topic is controversial. Extensive co-distribution and parallel organization of intermediate filaments and microtubules have been demonstrated in both flat and stellate cultured astrocytes (Goetschy *et al.*, 1986). Since IF distribution in cultured astrocytes can be changed by treatment with anti-microtubule drugs such as colchicine and vinblastine (Duffy *et al.*, 1982), and it is thought that this change in IF distribution is due to a loss of interaction with microtubules (Goldman *et al.*, 1979), it has been suggested that the cytoplasmic distribution of intermediate filaments is dependent on mirotubules (Goetschy *et al.*, 1986).

Treatment of cultured astrocytes with dB-cAMP causes a significant increase in GFAP and vimentin content, however alterations in cytoskeletal actin content are more closely correlated with changes in cell shape (Goldman and Chiu, 1984). Although the increase in GFAP and vimentin content in astrocytes was shown to be small after 1 week of treatment with dB-cAMP, the IF proteins had accumulated to 2-3 times the control level after the second week (Goldman and Chiu, 1984). This study indicates that changes in GFAP and

vimentin protein content require a substantial period of time to occur, and this may provide some insight as to why ultrastrucural changes due to acute activation of the HNS are more quickly reversed than ultrastructural changes due to chronic simulation of the HNS.

Cyclic AMP-mediated mechanisms also appear to regulate phosphorylation of GFAP and vimentin in astrocytes. β-adrenoreceptor activation can cause phosphorylation of GFAP and vimentin (Browning and Ruina, 1984; McCarthy *et al.*, 1985; Pollenz and McCarthey, 1986), and Goldman and Chiu (1984) have shown that increased cAMP concentration is associated with increased levels of phosphylation of both GFAP and vimentin. In addition to phosphorylation of GFAP and vimentin by cAMP and cAMP-dependent protein kinase mediated mechanisms, protein kinase C has been implicated to alter the amount of vimentin and GFAP proteins since phorbol esters and serotonin also enhance phosphorylation of both GFAP and vimentin (Pollenz and McCarthy, 1986). It is interesting to note that both GFAP and vimentin contain both unique and overlapping phosphorylation sites for both PKA and PKC (Harrison and Mobley, 1991).

Serum factors have also been shown to modulate astrocyte morphology and expression of IF proteins (Fischer *et al.*, 1982; Raff *et* al., 1983). Flat astrocytes cultured in serum-supplemented media have GFAP and vimentin intermediate filaments concentrated around the nucleus and dispersed in irregular arrays throughout the cytoplasm, whereas astrocytes in a serum-free medium have intermediate filaments oraganized as a radial fibrous network distributed from the nuclear region to the cells periphery (Goetschy *et al.*, 1986). Although there is evidence indicating that alterations in amounts of intermediate filaments proteins or phosphorylation status of GFAP and vimentin are associated with changes in cell

morphology, these intermediate filaments may not be required for changes in morphology. Pollenz and McCarthy (1986) were able to induce temperature-dependent morphological changes without phosphorylation of GFAP or vimentin, and Shain *et al.* (1987) have suggested that GFAP may not be critical for morphological changes since stellation occurs in LRM55 glioma cells which contain little GFAP.

B. Non-filamentous Cytoskeletal Proteins

Recent studies have implicated other cytoskeletal proteins such as focal adhesion kinase (FAK), paxillin, vinculin, and the small GTP-binding protein Rho in the regulation of cell morphology. FAK is a 125 KDa integrin-linked tyrosine kinase that associates with the Src and Fyn tyrosine kinases and paxillin (Schaller *et al.*, 1992; Schaller and Parsons, 1994). Paxillin is a cytoskeletal protein that binds vinculin, which is a cytoskeletal protein that is concentrated at the ends of stress fibers and at focal adhesions or sites of cell-substrate attachment (Burridge and Feramisco, 1980). Mammalian Rho proteins act on the actin cytoskeleton and have been shown to induce a rapid reorganization of actin into stress fibers in fibroblasts (Self *et al.*, 1993) and a variety of cell lines (Paterson *et al.*, 1990).

Serum and lysophosphatidic acid (LPA) have been demonstrated to stimulate actin stress fiber formation and focal adhesion assembly in fibroblasts (Ridley and Hall, 1992). LPA also stimulates tyrosyl-phosphorylation of cellular proteins, such as focal adhesion kinase and paxillin, which is accompanied by rapid recruitment of FAK, paxillin, vinculin, talin, as well as PKC-δ to focal adhesion sites (Ridley and Hall, 1994; Barry and Critchley, 1994; Chrzanwska-Wodnicka and Burridge, 1994). Microinjected active Rho mimics

exogenous LPA in recruiting tyrosyl-phosphorylated proteins to focal adhesion sites with subsequent stress fiber formation (Ridley and Hall, 1992, 1994). The exoenzyme C3 transferase from *Clostridium botulinum* which ADP- ribosylates and inactivates Rho proteins (Paterson *et al.*, 1990) completely inhibits LPA-induced stress fiber formation in serum starved Swiss 3T3 fibroblast cells (Ridley and Hall, 1992). These studies provide strong evidence that Rho proteins are involved in the regulation of the cytoskeleton.

Although there is an abundance of studies indicating that cAMP elevating agents can induce stellation in several cell types, there is a dearth of information on the pathway(s) connecting increased cAMP levels with alterations in cytoskeletal components. It has been demonstrated in non-astrocytic cells that cyclic nucleotides can influence tyrosyl-phosphorylation levels of cytoskeletal proteins. Han and Rubin (1996) demonstrated that cAMP can cause dephosphorylation of paxillin in rat Y1 adrenal cells, and Catalan *et al.*, 1995 have shown that forskolin and 8-br-cGMP can prevent endothelin-induced tyrosyl-phosphorylation of several substrate proteins in rabbit platelets. Although these studies were not performed in astrocytes, they do provide evidence that cyclic nucleotides can modulate tyrosyl-phosphorylation of cytoskeletal proteins.

In Swiss 3T3 cells, vasopressin (via a V1 receptor) has been shown to stimulate tyrosyl-phosphorylation of FAK and paxillin in a Ca^{+2} and PKC independent manner (Zachary *et al.*, 1993a and b). Although vasopressin has not been demonstrated to affect pituicyte morphology *in vitro* or *in vivo*, it is possible that small amounts of vasopressin could act on pituicytes via V1 receptors to cause tyrosyl-phosphorylation of FAK and paxillin which could help maintain engulfment of the neurosecretory terminals and

occupation of the basal lamina by pituicytes. This would therefore serve to prevent secretion of vasopressin into the systemic circulation until an overriding stimulus such as circulating epinephrine could induce retraction of pituicytes when the HNS was fully activated and vasopressin release was required.

A variety of agents appear to induce stellation or reverse stellation of cultured astrocytes. Although these agents must alter cytoskeletal proteins to change cell shape, exactly which cytoskeletal proteins are altered during these shape changes and the biochemical mechanisms mediating these effects are unknown. Studies presented here have focused on signal transduction pathways which mediate morphological changes in cultured astrocytes which are likely to mediate ultrastructural changes *in vivo*. Several agents alter astrocyte shape in culture, however there may only be a few which play a physiologically relevant role and induce ultrastructural changes *in vivo*. Which hormones or neurotransmitters are directly involved in alterations of the cytoskeleton and ultrastructural changes of astrocytes *in vivo* will require further investigation.

IX. Summary

Astrocytes in the central nervous system are a diverse population of cells, and we are only beginning to understand the critical roles that they play in regulating neural function. In the HNS, astroglial plasticity has been demonstrated in both the hypothalamus and the neurohypophysis, and it is likely that ultrastructural plasticity in the neurohypophysis regulates hormone secretion into the blood stream. There are a number of neurotransmitters and hormones which have the potential to modulate astroglial ultrastructure in the

neurohypophysis. Since the neurohypophysis is innervated by noradrenergic neurons, and circulating epinephrine and norepinephrine have direct access to pituicytes, there is the potential for these neurotransmitters to alter pituicyte ultrastructure by increasing intracellular cAMP levels in pituicytes *in vivo*. Several studies have demonstrated that β-adrenergic agonists and agents which increase intracellular cAMP concentration can induce stellation in astrocytes *in vitro*. Cultured pituicytes derived from the neurohypophyses of adult rats undergo morphological changes in response to β-adrenergic agonists, although the biochemical mechanism mediating β-agonist induced morphological plasticity in pituicytes remains to be defined.

Nitric oxide synthase has been localized in the neurohypophysis, and NOS inhibitors have been shown to attenuate osmotically-induced ultrastructural changes in the neural lobe. This strongly indicates that nitric oxide, and therefore cGMP, can regulate pituicyte morphology *in vivo*. Since atrial natriuretic peptide has been demonstrated in the neurohypophysis and ANP receptors have been localized on pituicytes, ANP appears to be a potential modulator of pituicyte ultrastructure *in vivo*. As ANP can activate particulate guanylate cyclase to elevate intracellular cGMP levels further indicates that cGMP may be involved in regulating pituicyte plasticity in the neural lobe.

Serum and lysophosphatidic acid (LPA) have been shown to reverse β-adrenergic induced stellation of astroglial cells *in vitro*. In addition, studies on cultured astroglial cells have shown that β-adrenergic agonists are less effective in inducing stellation in the presence of serum. Since the blood brain barrier is absent in the neurohypophysis, there is the potential for pituicytes to be exposed to serum and for serum to modulate pituicyte

ultrastructure *in vivo*.

Many cellular processes are critically dependent on intracellular calcium such as neurotransmitter release, and it is possible that alterations in intracellular calcium may be involved in regulating pituicyte plasticity. Calcium can alter intracellular cyclic AMP concentration in C6-2B glioma cells (Debernardi *et al.*, 1993), and ionophores which increase intracellular calcium concentration can cause stellation of astroglial cells (Frawthrop and Evans, 1987). Although it may appear contradictory, serum and LPA, which have been demonstrated to regulate the morphology of C6 glioma cells, can also stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate in a number of cells, which results in the mobilization of intracellular calcium and the activation of protein kinase C (van Corven *et al.*, 1989; van der Bend *et al.*, 1992). Since calcium has long been known to regulate actin polymerization and reorganization of the actin cytoskeleton (see Takai *et al.*, 1995), it is possible that alterations in intracellular calcium concentration are involved in regulating cytoskeletal changes in astroglial cells.

Recent studies on fibroblasts have indicated that serum can induce the formation of actin stress fibers and focal adhesions and cause tyrosyl-phosphorylation of certain cytoskeletal proteins, such as focal adhesion kinase and paxillin. Since serum can also regulate astroglial morphology in C6 glioma cells for example, it is possible that regulation of astroglial morphology occurs through tyrosyl-phosphorylation of cytoskeletal proteins.

X. Objectives

The objectives for the following studies are to test the following hypotheses concerning pituicyte and astroglial morphological plasticity. By determining the biochemical mechanisms mediating changes in astroglial morphology, we can further understand the significance of ultrastructural plasticity in astroglial cells in both normal brain function and in pathological states which may be due to faulty mechanisms which mediate astroglial plasticity.

1. Changes in pituicyte morphology are dependent on alterations in intracellular cAMP concentration. This hypothesis can be tested by directly activating adenylate cyclase, by inhibiting phosphodiesterase activity, or by applying cAMP analogs.

2. Changes in pituicyte morphology are dependent on changes in intracellular cGMP concentration. From this hypothesis, one may predict that cGMP analogs and agents which increase soluble or particulate guanylate cyclase activity or inhibit phosphodiesterase activity will alter pituicyte morphology.

3. Alterations in pituicyte morphology are dependent on the presence of serum. One may predict that serum will attenuate and reverse forskolin-induced stellation and will do so by one of the following mechanisms: 1) by acting through an inhibitory G-protein to inhibit adenylate cyclase activity, 2) by activation of a phosphodiesterase to decrease intracellular cyclic nucleotide concentration, or 3) by acting through an alternate signal transduction

pathway which eventually converges with the pathway mediating cAMP-induced stellation.

4. Alterations in intracellular calcium concentration modulate morphological plasticity in cultured pituicytes. This hypothesis may be tested by inhibiting influx of extracellular calcium or by depleting intracellular calcium stores.

5. Serum-induced morphological changes in astroglial cells are dependent on tyrosine kinase activity, and the status of tyrosyl-phosphorylation of cytoskeletal proteins is associated with astroglial morphology. One may predict from this hypothesis that inhibition of tyrosine kinase activity will alter the effects of serum on astroglial morphology.

METHODS

I. Pituicyte Cell Culture

The procedures for preparing explant cultures of pituicytes, treating the pituicyte cultures, and assessing the effects of the media on cell morphology have been adapted from methods originally described by Bicknell and colleagues (Bicknell *et al.*, 1989). Young adult male Sprague Dawley rats (175-200g; 60-80 days; Harlan) were euthanized by decapitation. The pituitary gland was rapidly removed and placed into sterile warm culture medium which consisted of Dulbecco's Modified Eagle Medium supplemented with newborn calf serum (10% v/v), HEPES (10 mM, pH 7.2), penicillin (100 U/ml), and streptomycin (100 μg/ml); (all materials for this medium were obtained from Gibco Life Technologies, Grand Island, NY). Under aseptic conditions, the neurohypophysis was isolated from each pituitary gland by removing the anterior and intermediate lobes (over 95% of the α-MSH activity is removed with this dissection - Bicknell *et at.*, 1983). Each neurohypophysis was cut into four approximately equally sized pieces. Individual explants were placed on sterile 22-mm diameter plastic coverslips (Lux Thermanox; Miles Laboratories, Naperville, IL) in sterile 35-mm tissue culture dishes. Explants were attached to the coverslips with clots formed from chicken plasma (0.5 mg/ml) and an equal volume of bovine thrombin (10.8 U/ml; Sigma Chemical Co, St. Louis, MO). Warm culture medium

(3 ml) was added to each dish, and the cultures were maintained in a humidified incubator (95% air, 5% CO_2) at 37°C for 14 days with one medium change at seven days.

II. Treatment of Pituicyte Cultures

After 14 days in culture, the explant-containing clot in each culture was removed, leaving a monolayer of cells adhered to the coverslip. The cultures were then rinsed twice with HEPES buffered salt solution (HBSS) supplemented with newborn calf serum (0.05% v/v). HBSS contains (mM); HEPES 10, NaCl 150, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, and D-glucose 5.6 (pH 7.4; 310 mOsmol/kg). Cultures were then incubated in appropriately modified HBSS experimental solutions (37°C, 95% O_2 and 5% CO_2 atmosphere) for treatment periods which depended on the type of experiment (stellation or destellation) and whether pretreatment periods were necessary.

The incubation time for pituicyte stellation experiments in which no pretreatment period was required was 90 min. A 90 min incubation was used in most pituicyte experiments because it provides ample time for stellation-inducing agents to cause stellation while allowing most non-stellate pituicytes incubated in HBSS to remain non-stellate. In some experiments, test substances were added to the experimental solutions during the treatment period and to the experimental solution or cultured medium in a pretreatment period. These are detailed in each of the relevant experimental sections.

Pituicyte cultures in destellation experiments were incubated for two consecutive 90 min incubation periods so that cultures were incubated for a total of 180 min. The first 90 min incubation period was in an experimental medium containing a stellation-inducing agent

such as forskolin. The pituicyte cultures were then subsequently incubated for 90 min in an experimental solution containing test substances so that it could be determined whether a particular agent could induce or prevent destellation. Following treatment in the experimental medium, each culture was fixed (30 min; 21 °C) with 4% paraformaldehyde and 0.2% picric acid in phosphate buffer (0.15 M; pH 7.6) and then rinsed 3 times in and stored (at 4 °C) in HBSS. Drugs which were used in pituicyte studies are listed in Table 1.

III. RG-2 Cell Culture

RG-2 cell line cultures were originally isolated from the cerebral cortex of embryonic rats (Ko *et al.*, 1980) and were obtained at passage numbers P11x-P16x from Dr. Diane Matesic. RG-2 cells were grown in culture medium which consisted of Dulbecco's Modified Eagle Medium supplemented with (mM) Na pyruvate 1.25, glucose 5.55, NaCl 14.3, $NaHCO_3$ 11.9, and 5% v/v fetal calf serum. When RG-2 cells were confluent on the bottom of a culture flask, the culture medium was removed, and the cells were trypsinized for 20 min in 5 ml EBSS + 0.05% trypsin (Sigma). Culture medium (6 ml) was then added to the flask, the cells were triturated, and 10 ml of cells were transfered to to a conical centrifuge tube and centrifuged at 1000 g for 10 min. The medium was removed and 1 ml culture medium was added to the pellet. After trituration, 100 µl of the cell suspension was transfered to a 50 ml centrifuge tube and 20 ml of culture media was added to the 100 µl of cells. The cells were diluted to a density of 7×10^4 cells/ml. Some of the cell suspension (250 µl) was placed onto each of 24-48 sterile 22-mm diameter plastic coverslips in sterile 35-mm tissue culture dishes and incubated in a humidified atmosphere (37 °C; 95% air; 5% CO_2) for 4 hr to allow

Table 1 - Drugs used for pituicyte studies

ANP	Peninsula	binds ANP receptor to increase $[cGMP]_i$
8-bromo-cAMP	RBI	cAMP analog
8-bromo-cGMP	RBI	cGMP analog
Dideoxyforskolin	Sigma	non-cyclase activating forskolin analog
Forskolin	RBI	direct activator of adenylate cyclase
Fura-2	Molecular Probes	intracellular calcium indicator dye
Genistein	Sigma	tyrosine kinase inhibitor
H-7	RBI	protein kinase inhibitor (PKA selective)
H-89	Calbiochem	protein kinase inhibitor (PKA selective)
IBMX	Sigma	non-specific phosphodiesterase inhibitor
Isoproterenol	Sigma	beta adrenergic agonist
LY-83,583	RBI	soluble guanylate cyclase inhibitor
Methylene Blue	Sigma	soluble guanylate cyclase inhibitor
Milrinone	Sigma	cGMP-inhibited PDE (PDE 3) inhibitor
My-5445	Calbiochem	cGMP-specific PDE (PDE 5) inhibitor
Pertussis Toxin	Sigma	Gi-protein inhibitor
Rp-cAMPS	RBI	PKA inhibitor
SIN-1	Molecular Probes	nitric oxide donor
Sodium Nitroprusside	Sigma	nitric oxide donor
Thapsigargin	RBI	depletes intracellular calcium stores

* Calbiochem, La Jolla, CA
* Molecular Probes Inc., Eugene. OR
* Pensinsula Labs, Belmont, CA
* Research Biochemicals (RBI), Natick, MA
* Sigma, St. Louis, MO

adherence of cells to the coverslips. Culture medium (3 ml) was then carefully added to each dish of cells, and the cultures were incubated for 10 days (37°C; 95% air; 5% CO_2) with a medium change at 5 days.

IV. Treatment of RG-2 Cells

After 10 days in culture, a monolayer of RG-2 cells was observed in each dish, and cultures were rinsed twice with HEPES buffered salt solution (HBSS) supplemented with 0.05% newborn calf serum. Cultures for stellation experiments were incubated in experimental solutions (HBSS appropriately modified) at 37°C for 60 minutes (a preliminary experiment was done to determine an optimal incubation period that would allow stellation-inducing agents to cause stellation while leaving most RG-2 cells incubated in HBSS non-stellate). RG-2 cell cultures for destellation experiments were incubated for 60 min in HBSS supplemented with forskolin (5 μM) to induce stellation and then either fixed (controls) or subsequently incubated for 20 min in a second experimental medium. Cultures for morphology experiments were then rinsed once with HBSS, fixed in 4% paraformaldehyde containing 0.2% picric acid in phosphate buffer (0.1 M, pH 7.4) for 30 minutes, and then rinsed in HBSS 3 times and stored in HBSS (4°C) until RG-2 cell morphology could be assessed. RG-2 cell cultures which were to be used for Western Blot analysis were rinsed with 2 ml PBS and lysed with ice cold buffer containing: Tris HCl, 50 mM; NaCl, 155 mM; EGTA, 2 mM; Triton X-100 (0.1%); phenylmethylsulfonyl fluoride (PMSF; Sigma), 1mM; sodium-orthovanadate (Sigma), 1mM; and the microbial protease inhibitor leupeptin (Sigma), 5 mg/ml. Lysates from the four dishes within the same experimental treatment

group in an experimental replicate were pooled (400 µl total volume) and centrifuged (10,000 g, 10 min, 4°C), and the supernatants (300 µl) were frozen at -80°C until Western Blot analysis could be performed.

V. Evaluation of Cell Morphology

The morphology of fixed pituicyte and RG-2 cell cultures was examined using phase contrast optics. Four separate fields of cells (each 1 mm^2 area) from the outer edge of the pituicyte or RG-2 cell monolayers were examined. Pituicytes and RG-2 cells were judged to be stellate if they possessed a clearly defined, phase bright cell body with one or more distinct, phase bright processes. Cells were classified as non-stellate if they had neither a well defined soma nor distinct processes. These parameters for evaluating cell morphology were adapted from methods described by Bicknell *et al.* (1989). In each field, the numbers of stellate cells and non-stellate cells were determined, and the counts for all four fields in a single culture were pooled. The fraction of stellate cells in each culture was determined by calculating the ratio of the number of stellate cells to the total number of cells counted.

To eliminate experimenter bias, these studies were performed blind. Each experimental medium was coded prior to use so that treatment of the cultures was performed blind. In addition, cultures were coded immediately after fixation so that morphological evaluation of cells in each culture was blind to the treatment group assignment of each culture. The treatment of each culture remained unknown until after the procedure for assaying cell morphology for all cultures within every replicate of an experiment was complete. Within each of 2-4 replicates of each experiment there were 3 or 4 cultures

treated with each experimental medium. Therefore in each experiment there were 8-12 cultures treated with each experimental medium. The mean and standard error of the mean for each treatment group for each experiment are represented in graphical figures. The Kruskal Wallis test with appropriate post-hoc corrections (Crunch 4 Statistical Analysis) was used for detection of statistically significant effects of the various media on pituicyte and RG-2 morphology.

VI. Immunocytochemical Procedures

In order to demonstrate that morphologically plastic cells from pituicyte cultures were truely astrocytic pituicytes, some cultures were stained for the astrocyte marker glial fibrillary acidic protein (GFAP) (Salm *et al.*, 1982). Previously fixed pituicyte monolayers were rinsed twice with 0.1 M phosphate buffered (pH 7.4) 0.15 M NaCl (PBS) and then incubated in 2% goat serum (Chemicon International Inc., Temecula, CA) at room temperature for 60 min (to prevent non-specific staining of cultures with the fluorescent secondary antiserum). Pituicyte cultures were then incubated (48 hr, 4°) in a polyclonal primary antibody against GFAP (1:400; Chemicon). Cultures were rinsed with PBS, incubated in indocarbocyanine-conjugated (Cy3™) rabbit anti-goat IgG secondary antiserum (90 min, 21°C; 1:1000; Jackson Immnoresearch Labs.), and then rinsed again in PBS. All sera were diluted in a proprietary buffer (Superblock™ Blocking Buffer in PBS, Pierce Chemical Co., Rockford, IL USA) designed to reduce non-specific binding of antisera. Pituicyte cultures were placed upside down in Tris buffer (pH 8.6) or buffered glycerol (pH 8.6) on a glass coverslip. Cultures were visualized using an inverted Nikon diaphot

microscope equipped with an epifluorescence collector lens, and Cy3™ was visualized using a barrier cube (dichroic mirror 580 nm, 546 excitation filter, and 590 nm barrier filter).

VII. Perfusion Procedure for Determining Rates of Pituicyte Stellation and Destellation

Pituicyte cultures were prepared from adult male rat neurohypophyses as previously described, except that neurohypophysial explants were plated on the bottom of 35 mm tissue culture dishes instead of onto plastic coverslips so that pituicyte monolayers would be adhered directly to the bottom of the dish. This was done to avoid movement of the coverslip during perfusion and to ensure that the same field of cells could be monitored throughout the 180 min perfusion period. After 14 days in culture, the explants were removed and each culture was rinsed twice with 0.05% NCS supplemented HBSS. Dishes containing pituicyte monolayers were mounted on the stage of a Nikon TMS microscope, and cultures were perfused (perfusion rate = 1 ml/min) with warm HBSS for a 3 min equilibration period. Cultures were perfused with HBSS containing forskolin (5 μM) for 90 min to induce stellation of pituicytes, and photographs were taken every 5 minutes, using Kodak Tech Pan film with a Nikon N-2000 camera, so that the rate of stellation in response to 5 μM forskolin could be determined. Pituicyte monolayers were subsequently perfused with HBSS for 3 min to remove the forskolin-containing HBSS solution remaining in the dish and therefore to remove the stellation-inducing agent. Cultures were then rinsed with HBSS supplemented with 0.5% NCS for 90 min. Photographs were taken every 5 min so that the rate of destellation in response to perfusion of pituicytes with 0.5% serum could be determined. Film was developed with Kodak HC-110 developer for 7 min 30 sec, fixed for

7 min 30 sec, and then rinsed in a solution containing Photo-flo (Kodak, Rochester, NY). The negatives were dried on medium heat for 45 min and then visualized with a light box so that the rates of stellation and destellation of pituicytes could be subjectively determined.

VIII. Measurement of Intracellular Ca^{+2}

Fluorescent probes, such as fura-2, have been used in cell biology to indicate the location and quantity of cellular structures, and for fluorescent molecules which are sensitive to particular aspects of their environment, to allow monitoring of a parameter of interest and changes of this parameter with various cellular manipulations. Fluorescence is a molecular process whereby absorption of radiation from a source results in emission of electromagnetic radiation. Fura-2 is a calcium indicator dye with a high fluorescent yield, and this property allows one to use fura-2 to examine $[Ca^{+2}]i$ at the single cell level, as has been done in this study. Use of fura-2 in this manner is advantageous in several aspects. Single-cell Ca^{+2} measurements circumvent problems associated with heterogeneous cell populations and allow data to be obtained from an identifiable cell type and eliminate contributions from dead, damaged, or foreign cells. Although cell population measurements are easier to carry out and the signal-to-noise is often better, single-cell analysis provides the most accurate results and is the most reaonable approach for this particular type of study.

To allow non-disruptive loading of Ca^{+2} indicators into cells, intact cells can be incubated with an esterified form of the indicator (e.g. fura-2 AM). Esterified esters are uncharged and hydrophobic and therefore readily cross cell membranes. Once inside the cell, endogenous esterases can activate the Ca^{+2} indicator dye and release the free acid form

which is not permeable to the cell membrane. This allows accumulation of the indicator dye in the cell cytosol, and once the extracellular Ca^{+2} has been removed, the fluorescent signals from the cytosolic dye can be obtained and used to calculate the intracellular [Ca^{+2}].

When Ca^{+2} binds to fura, the nitrogen lone pair of electrons is withdrawn from the aromatic rings resulting in altered fluorescence properties (Tsien, 1989). The changes in fluorescence may be used to calculate the proportion of fluorescent dye in the calcium-bound and calcium-free forms. To ensure that the largest possible signal changes are obtained for given changes in Ca^{+2} concentration, it is important that the binding affinity of the indicator be in the same range as the free [Ca^{+2}] being measured (Thomas and Delaville, 1991). Although Ca^{+2} indicator dyes such as fura-2 are selective for Ca^{+2} over Mg^{+2}, several other ions, such as Mn^{+2}, Zn^{+2}, Fe^{+2} and Co^{+2} can bind the fluorescent indicators and can interfere with calibration of the dyes (Thomas and Delaville, 1991).

There is usually one or more optimal wavelengths for excitation which coincide with a maximum absorption and a related optimum wavelength of emission. By using dual wavelength excitation (eg. 350 nm and 380 nm), the proportion of the fluorescent dye can be measured in one of two forms (eg. Ca^{+2}-bound fura-2 vs free fura-2) from the ratio of fluorescence measured at the two wavelengths. This particular method is extremely valuable because the ratio calculated from measurement of the intensity of light emitted at 505 nm in response to excitation at 350 nm and 380 nm is independent of dye distribution or concentration.

Pituicyte cultures were prepared from neurohypophysial explants as previously described. After 14 days in culture, the explant-containing clots were removed from each

culture and the remaining pituicyte monolayers were rinsed twice with serum-free HBSS. Fura-2 aminodiester (9µl) (Molecular Probes Inc., Eugene, OR) in DMSO (1 mg/ml) was diluted in 3 ml of warm HBSS, and pituicytes were passively loaded with 3 µM fura-2 AM for 45 min in a humidified incubator (37°C, 95% air, 5% CO_2). A coverslip on which pituityes were attached was placed into a perfusion chamber and mounted on the stage of a Nikon diaphot-TMD microscope. The perfusion chamber was filled with warm HBSS and the cultures were rinsed in the dark for 30 min at a perfusion rate of 1 ml/min (15 min with HBSS supplemented with 0.5% NCS followed by 15 min with HBSS alone) to remove extracellular Fura-2. Pituicytes, which were not touching each other, were visualized through a Nikon quartz 40 x oil-immersion epifluorescence objective, and fura-2 fluorescence was optimized during the rinsing period.

Changes in intracellular Ca^{+2} concentration were determined by calculating the ratio of calcium-bound fura-2 to free fura-2. This ratio was determined by measuring the emission wavelength of 505 nm in response to excitation at wavelengths of 350 nm and 380 nm and calculating the ratio. Excitation wavelengths (350 nm and 380 nm) were selected by means of a computer-controlled rotating filter wheel between a xenon lamp, which served as a light source, and the microscope. The emission light at 505 nm was passed to an image-intensifying charge-coupled device camera. The resulting image from each excitation wavelength was processed and stored, and the time resolution was set at 6 sec between ratio frames during the baseline perfusion and then at 4 sec between ratio frames for the subsequent perfusion periods for each experiment. The Ionoptics system was used to collect data, and the Ion Wizzard program was used for image processing. Background images at

350 nm and 380 nm were captured at the beginning of each experiment so that the background fluorescence could be subtracted by the computer software. The background fluorescence images were taken from an area of the coverslip containing no cells or debris. Both background images (350 nm and 380 nm) were subtracted from their relevant experimental images at the same wavelengths at the end of each experiment. The ratio of light intensities emitted from calcium-bound fura-2 and free fura-2 in response to excitation at 350 nm and 380 nm respectively was calculated for each frame from the equation of Grynkiewicz *et al.* (1985): $[Ca^{+2}]_i = K_d \times (R-R_{min})/(R_{max}-R) \times (S_{f2}/S_{b2})$, where K_d is the dissociation constant for Fura-2/Ca^{+2}, R_{max} is the 350/380 nm ratio during Ca^{+2} saturation, R_{min} is the 350/380 nm ratio during Ca^{+2}-free conditions, and S_{f2} and S_{b2} are the emission intensities at 380 nm excitation during Ca^{+2}-free and Ca^{+2}-saturating conditions respectively. The calibration constants R_{max} and R_{min} were determined to be 2.50 and 0.50 respectively, S_{f2} and S_{b2} were determined to be 150 and 40 respectively, and the K_d was 224. Collected data was analyzed by the Ionoptics computer software, and fluorescence ratios and traces were displayed graphically using the Ion Wizzard program.

IX. Western Blot Analysis

RG-2 cell cultures which had been prepared for Western Blot analysis and frozen were thawed to room temperature. Each lysate (80 µl) was prepared with denaturing sample buffer (20 µl) and boiled for 5 min. RG-2 cell lysates for one experiment were then loaded (20 µl per lane) onto a 10% denaturing sodium dodecyl sulfate (SDS) polyacrylamide gel and electrophoretically separated (150-200 V, 45 min). To prepare Immobilon-P (polyvinyl

difluridine; Millipore Bedford, MA) membranes for protein transfer, the membranes were soaked in methanol for 5 sec, then soaked in water for 5 min, and then temporarily stored in transfer buffer. Proteins were electrically transferred from the gel to a prepared Immobilon-P membrane (100 V, 1 hr, 4°C). A successful protein transfer was indicated by the transfer of rainbow molecular weight standards (Amersham Life Science), which were run along side the lysate samples. Membranes were incubated (4°C, 4 hr) in blocking buffer (Tris-buffered (200 mM, pH 7.6) saline (NaCl 137 mM) supplemented with Tween-20 (0.1%), 4% chicken egg ovalbumin (Sigma), and 0.025% sodium azide, (Sigma)) to prevent non-specific binding of antisera. Membranes were then incubated overnight (4°C) in mouse phosphotyrosine antibody (Upstate Biotechnology Inc., Lake Placid, NY) in blocking buffer (1:7500). After three rinses with Tris-buffered saline + Tween (0.1%) (30, 5, and 5 min) and one rinse with Tris-buffered saline (5 min), membranes were incubated for one hour (4°C) in a secondary antiserum (sheep anti-mouse IgG; Amersham Life Science, Buckinghamshire, England) linked to horseradish peroxidase diluted (1:7500) in Tris-buffered saline. Membranes were rinsed as previously described, and proteins which were labeled with the phosphotyrosine antibody were visualized using enhanced chemiluminescence (Amersham Life Science) and images were saved on film. The membrane blots were then immersed in a solution of 100 mM β-mercaptoethanol and 2% SDS in 62.5 mM Tris HCl (pH 6.7) at 50°C for 30 min with agitation, and washed (2 x 10 min in TBS-T) - this procedure removed the previous antibodies so that the membrane blots could be reblocked in blocking buffer and reprobed with another antibody. The membranes were then stained immunocytochemically, using the same procedure as described previously, for MAP (mitogen activated protein) kinases using

a monoclonal mouse antiserum against Erk 1 and Erk 2 (extracellular signal-regulated kinase) (1:5000; Zymed Laboratories, San Francisco, CA). Autoradiographic films (Reflection NEF autoradiography film; Du Pont, Boston, MA) from stained membranes were scanned into a computer, and densitization of protein bands of selected molecular weights using the Molecular Analyst 2.1 (Biorad) and Adobe Photoshop (Adobe Systems Inc.) programs allowed comparison of staining densities across treatment groups.

EXPERIMENTAL STUDIES

I. Astroglial Morphology and Immunocytochemistry

A. Neurohypophysial Explants and Pituicyte Identification

The methods for preparing neurohypophysial explant cultures was adapted from the procedure first described by Bicknell *et al.* (1989). Within 3-4 days after plating the neurohypophysial explants, an outgrowth of cells from the explants could be observed (Figure 3A). More cellular outgrowth could be visualized by approximately 10 days in culture (Figure 3B), and by 14 days in culture a monolayer of cells beyond the boundary of the explant-containing clot had been established (Figure 3C). Pituicyte cultures of 14 days of age were used for all pituicyte experiments. In untreated cultures which were removed from culture medium, briefly rinsed twice with warm HBSS, and then immediately fixed, the predominant cell type was one with a flattened, irregular morphology with some cells having one or more flattened processes (nonstellate) (Figure 4A). Incubation of pituicytes in warm HBSS containing the β-adrenergic agonist isoproterenol (20 μM) induced pituicytes to display a stellate morphology, with each cell having a phase-bright rounded cell body and one or more phase-bright, finely branching processes (Figure 4B). Observation of individual cells has demonstrated that the morphological response to treatment of pituicytes with HBSS containing isoproterenol or forskolin consists of retraction of cytoplasm from the cell

Figure 3. Pituicyte monolayers are derived from cultured neurohypophysial explants. Using phase-contrast microscopy, pituicytes can be observed migrating out from a neurohypophysial explant within 3-4 days in culture (A). Pituicyte outgrowth is more extensive by approximately the 10th day in culture (B), and a pituicyte monolayer is established after being in culture for 14 days (C).

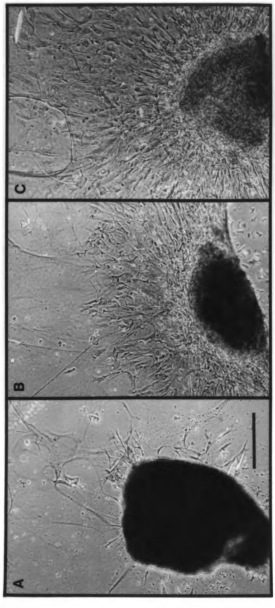


Figure 3

Figure 4. Pituicyte morphology is plastic in culture. Phase contrast photomicrographs illustrate that cultured (14 days) pituicytes incubated for 90 min in HEPES buffered salt solution (HBSS) are mostly non-stellate (A), and cultures incubated in HBSS supplemented with isoproterenol (20 μM) are mostly stellate (B) (Scale bar represents 100 μm).

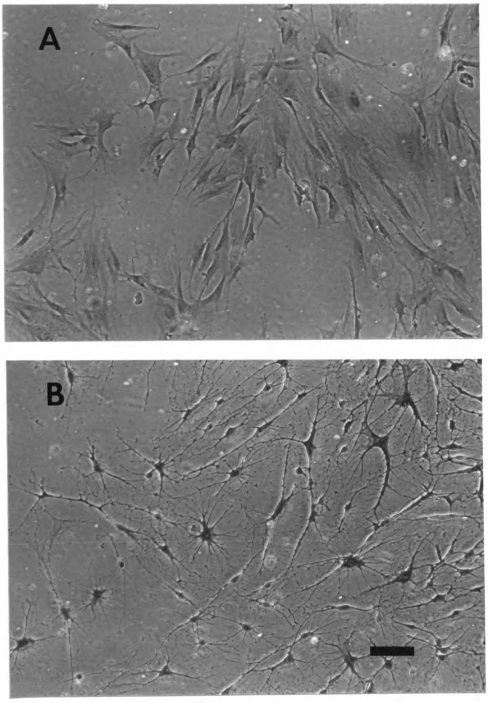


Figure 4

periphery into the perinuclear area to form a prominent cell body with branching processes.

As mentioned previously, SON projections terminate selectively in the central part of the neurohypophysis, whereas PVN projections terminate selectively in the more peripheral regions (Alonso and Asenmacher, 1981). The selectivity of the axon terminations should have had no influence on the pituicyte studies presented in this paper for three reasons. First, each neurohypophysis was cut into four equally sized pieces so that each explant provided pituicytes which had migrated from both central and peripheral regions of the neurohypophysis. Second, most axons within the neurohypophysis should have had little or no influence on pituicytes during culturing since they would have degenerated by 14 days in culture, which is when pituicytes are normally used for experimental procedures. And third, the clots containing the magnocellular axons and axon terminals were removed prior to pituicyte experiments, therefore removing all potential neuronal influences on pituicytes.

Early studies of cultured cells relied mainly on morphological criteria to identify astrocytes. As this method of identification was often not definitive, the isolation and characterization of the astrocyte specific intermediate filament protein glial fibrillary acidic protein (GFAP) marked a significant advancement in the study of these cells (Eng *et al.*, 1971). The development of antibodies to GFAP (Uyeda *et al.*, 1972; Dahl and Bignami, 1973) has enabled investigators to use immunocytochemical techniques to identify astrocytes both *in situ* (Uyeda *et al.*, 1972; Dahl and Bignami, 1973) and *in vitro* (Antanitus *et al.*, 1975; Stieg *et al.*, 1980). The presence of GFAP in pituicytes *in situ* (Salm *et al.*, 1982; Suess and Pliška, 1981) and in *vitro* (Bicknell *et al.*, 1989) has been independently described. In the present studies, some cultures were stained for GFAP to ensure that the

morphologically plastic cells were indeed astrocytic pituicytes. Non-stellate cells stained diffusely for GFAP when treated with a GFAP-antiserum (Figure 5), whereas stellate cells stained relatively intensely for GFAP (Figure 5). This difference in staining intensity is not necessarily due to alterations in amounts of GFAP. Since cytoplasmic retraction decreases the amount of contact between pituicytes and the coverslip to which they are adhered, the thickness of the pituicytes therefore increases and stellate pituicytes appear to stain more intensely for GFAP than non-stellate pituicytes.

As described in detail in subsequent sections, certain test substances induce pituicytes to become stellate and others induce stellate pituicytes to revert back to a non-stellate form. It was of interest to observe stellation and destellation as a function of time. To determine the rate of stellation and the rate of destellation, pituicyte cultures were perfused with warm HBSS containing 5 μM forskolin for 90 minutes at a rate of 1 ml/min to induce stellation, and then perfused with warm HBSS supplemented with 0.5% newborn calf serum (NCS) at 1 ml/min for a subsequent 90 min period to induce destellation. A small amount of cytoplasmic retraction of pituicyte cytoplasm could be observed within the first 15 minutes of perfusion with HBSS containing forkolin, and the cells appeared to be stellate after approximately 1 hr (Figure 6). It was apparent that stellate pituicytes were beginning to revert to a non-stellate morphology as soon as 5-10 min after being perfused with HBSS supplemented with 0.5% NCS, and the pituicyte cultures appeared to have returned to a non-stellate morphology within 30 min (Figure 7). This experiment may appear to suggest that stellation occurs more slowly than destellation. However, rates of stellation and destellation are likely to be concentration-dependent as well as time-dependent, and since only one

Figure 5. Pituicytes cultured from the neurohypophysis are astrocytes. Phase contrast (A and C) and epifluorescence optics (B and D) photomicrographs of pituicytes in culture derived from a neurohypophysial explant illustrate that most cells stained positive for the astrocytic marker glial fibrillary acidic protein using a primary polyclonal antiserum against GFAP and a Cyanine 3 fluorescent labeled secondary antiserum. Non-stellate cells stain diffusely for GFAP (B) relative to stellate cells (D) (Scale bar represents 100 μm).

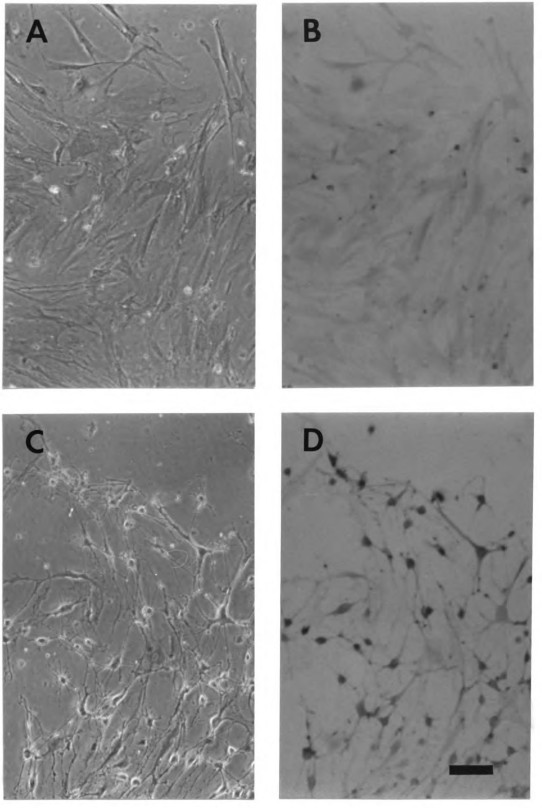


Figure 5

Figure 6. Determination of the rate of stellation of cultured pituicytes. Phase contrast optics show that perfusion (1 ml/min for 90 min) of cultured pituicytes with HBSS containing forskolin (5 μM) causes stellation to occur progressively. Cells are initially non-stellate (A), but they become relatively more stellate after 15 min (B), 30 min (C), 45 min (D), 60 min (E), and 75 min (F) of perfusion with 5 μM forskolin in HBSS.

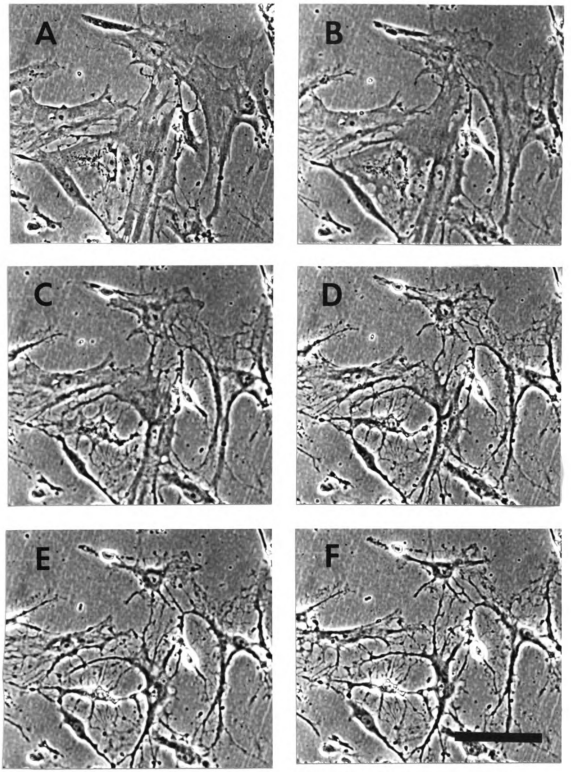


Figure 6

Figure 7. Determination of the rate of destellation of pituicytes in culture. Using phase contrast optics, stellate pituicytes (initially perfused with HBSS containing forskolin (5 μM) for 90 min) are shown to revert back to a non-stellate form. Stellate pituicytes appeared to revert from a stellate mophology (0 min; A) back to a non-stellate form within about 10 min when perfused with HBSS containing serum (0.5%). Pituicyte morphology is shown after 10 min (B), 20 min (C), 30 min (D), 40 min (E), and 50 min (F) of perfusion with HBSS supplemented with 0.5% serum.

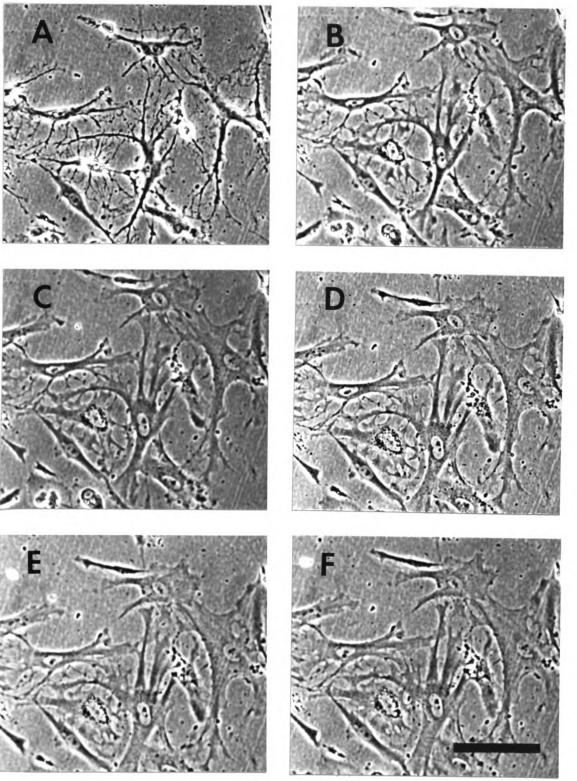


Figure 7

Figure 8. RG-2 cells are plastic in culture. Phase contrast photomicrographs demonstrate that cultured (10 days) RG-2 cells incubated for 60 min in HEPES buffered salt solution (HBSS) are mostly non-stellate (A), and cells incubated in HBSS supplemented with forskolin (5 μM) for 60 min are mostly stellate (B) (Scale bar represents 100 μm).

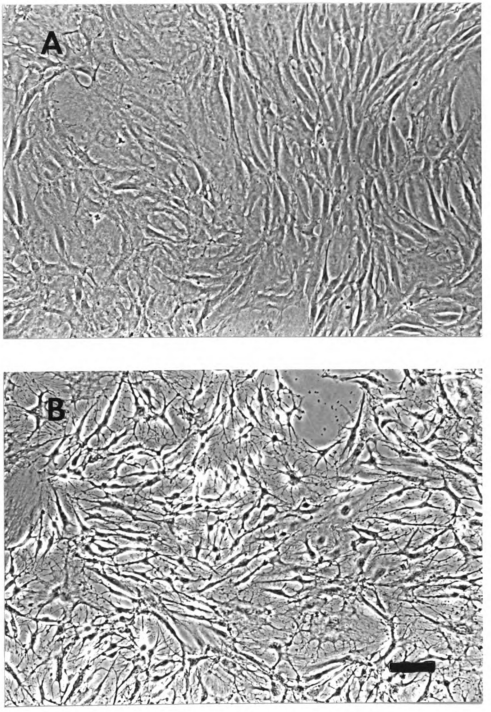


Figure 8

concentration of each test substance was used, rates for stellation and destellation cannot be compared.

B. RG-2 Cell Morphology

As described later, Western Blot analysis requires more protein than can be obtained from pituicyte cultures. Therefore RG-2 cells, which provide an ample quantity of protein, were used in both morphology and Western Blot analysis experiments. RG-2 cell line cultures which had originally been isolated from the cerebral cortex of embryonic rats (Kumar *et al.*, 1993) were used at passage numbers of P11x-P16x for RG-2 cell experiments. After being plated on coverslips and grown in culture for 10 days, RG-2 cell experiments were performed. An initial experiment was done (data not shown) to determine an optimal incubation period for stellation of RG-2 cells to occur in response to inducing agents while allowing cultures incubated in only HBSS to remain non-stellate. RG-2 cells remained non-stellate when incubated in only HBSS for 60 min (Figure 8A), but RG-2 cells incubated in HBSS containing forskolin (5 μM) for 60 min assumed a stellate morphology (Figure 8B).

II. cAMP Mediates Stellation of Cultured Pituicytes

A. Introduction and Experimental Rationale

Many studies have indicated that increased intracellular cAMP concentration is involved in mediating morphological alterations in primary cultured astrocytes and astroglial cells. When astrocytes in primary cell culture (Bradford, 1976; Haugen and Laerum, 1978; Federoff *et al.*, 1984; Narumi *et al.*, 1978; Pollenz and McCarthy, 1986) or C-6 glioma cells (Oey, 1975) are treated with β-adrenergic agonists, activators of adenylate cyclase, or cAMP analogs, the cells change from an epithelial-like to a stellate morphology. β-adrenoreceptor activation has been shown to result in cAMP synthesis in primary astrocyte cultures (deVellis and Brooker, 1972; Ebersolt *et al.*, 1981; Shain *et al.*, 1986) and in C-6 glioma cells (Browning *et al.*, 1976; Opler and Makman, 1972). Although it has been proposed that morphological changes in glial cell line cultures or in astrocyte cultures derived from neonatal rat brain result from activation of cAMP-dependent protein kinase (PKA) (Browning and Ruina, 1984; McCarthy *et al.*, 1985), the role of cAMP and PKA in mediating these morphological alterations has not been thoroughly examined.

Cultured pituicytes derived from adult rat neurohypophyses have also been shown to change morphologically from a flat epithelial-like shape to a stellate, process-bearing form in response to treatment with norepinephrine or isoprenaline (Bicknell *et al.*, 1989), and this response has been shown to occur via activation of β_2-adrenoreceptors (Hatton *et al* 1991). Although the involvement of cAMP and cAMP-dependent protein kinase in regulation of pituicyte morphology has not been previously investigated, several physiologically relevant implications have demanded that this topic be addressed. For example, NaCl-treated and

lactating rats have increased neurohypophysial cAMP concentration compared to non-treated and non-lactating controls (Ruoff *et al.*, 1976). Also, adrenal medullectomy prevents NaCl-induced neurohypophysial ultrastructural changes in rats, indicating that circulating epinephrine may act at β-adrenergic receptors on pituicytes to induce cytoplasmic retraction *in vivo* (Beagley and Hatton, 1994). In addition, a cytochemical reaction product for adenylate cyclase has been localized to pituicytes of the neurohypophysis (but it is absent in intermediate lobe cells) (Santolaya and Lederis, 1980), which indicates that the biochemical machinery to produce cAMP is present and further implicates cAMP as a second messenger involved in pituicyte structural or functional regulation. Understanding biochemical pathways which mediate stellation of cultured pituicytes *in vitro* may give some insight as to how pituicyte ultrastructure is modulated *in vivo*. Future studies that determine which agents induce ultrastructural changes *in vivo* and which biochemical mechanisms mediate these changes will hopefully help explain what role pituicytes play in regulating neurohypophysial hormone release.

In many cell types, stimulation of β-adrenergic receptors with the catecholamines epinephrine or norepinephrine results in profound increases in intracellular cAMP concentration. Detectable increases in the concentration of cAMP occur within a few seconds of exposure to an agonist, and in some cases cAMP levels can increase up to 400-fold over basal within minutes (Shear *et al.*, 1976; Johnson *et al.*, 1978). Three plasma membrane proteins required for an increase in intracellular cAMP to occur are the β-adrenergic receptor, the stimulatory G protein (Gs), and adenylate cyclase, which is the enzyme which catalyzes the formation of cAMP from ATP. The interaction of the agonist-

β-adrenoreceptor complex with Gs facilitates the release of GDP from the α subunit of the G protein (αs) allowing the binding of GTP and the subsequent direct activation of adenylate cyclase by αs-GTP (Levitzki, 1988). Adenylate cyclase is regulated by a stimulatory G protein (Gs) and an inhibitory G protein (Gi), and intracellular cAMP levels are regulated by several processes such as the generation of cAMP from ATP by adenylate cyclase, the degradation of cAMP by phosphodiesterases, and the export of cAMP out of the cell (see Hausdorff *et al.*, 1990).

The involvement of adenylate cyclase and intracellular cAMP in modulation of pituicyte morphology has been investigated in the following studies to help elucidate the biochemical pathways which mediate these morphological changes. The role of cAMP in regulating pituicyte morphology was examined by activiting or inhibiting biochemical pathways known to modulate intracellular cAMP concentration in cells. In particular, the effect of inhibition of the inhibitory G protein (Gi), activation of adenylate cyclase, inhibition of phosphodiesterases, elevation of intracellular cAMP concentration with cAMP analogs, and inhibition of cAMP-dependent protein kinase was investigated in relation to pituicyte morphology.

B. Results

1. Stellation of Cultured Pituicytes is Induced by β-adrenoreceptor Activation, by Direct Activation of Adenylate Cyclase, and by the cAMP Analog 8-bromo cAMP

Cultured pituicytes change from a flat, epithelial-like shape to a stellate, process-bearing form when incubated for 90 minutes in HEPES buffered salt solution (HBSS)

containing the β-adrenergic agonist isoproterenol (1-[3'4'-Dihydroxyphenyl]-2-isopropyl-aminoethanol) compared to cultures incubated in only HBSS as shown in Figure 9. HBSS containing 5 μM forskolin (7β-Acetoxy-8,13-epoxy-1α,6β,9α-trihydroxy-1abd-14-ene-11-one), which is a diterpine known to directly activate adenylate cyclase (Awad *et al*, 1983; Seamon and Daly, 1981), also induces cultured pituicytes to become stellate (Figure 9), and it does so in a concentration-dependent manner (Figure 10). Since incubation of pituicyte cultures in HBSS containing 1,9 dideoxyforskolin (5 μM; 7β-Acetoxy-6β-hydroxy-8,13-epoxy-1abd-14-en-11-one; Sigma), which is an inactive forskolin analog which does not activate adenylate cyclase, had no significant effect on pituicyte morphology (Figure 9), it is probable that forskolin does induce stellation via activation of adenylate cyclase. Dimethyl sulfoxide, the vehicle for forskolin and 1,9 dideoxyforskolin, also had no effect on pituicyte morphology (Figure 9).

Once it had been established that direct activation of adenylate cyclase could induce stellation of cultured pituicytes, the cAMP analog 8-bromo cAMP (8-bromo-adenosine cyclic 3'5'-hydrogen phosphate monosodium salt) was used to investigate if increasing intracellular cAMP concentration would induce stellation of pituicytes in culture. HBSS containing 8-bromo cAMP (150 μM) induced a significant fraction of pituicytes in culture to become stellate compared to pituicyte cultures incubated in HBSS alone (Figure 11). Since cAMP is predominantly known to activate cAMP-dependent protein kinase (PKA), it would be reasonable to assume that cAMP activates PKA to mediate stellation, although subsequent findings (discussed later) challenge this assumption.

Figure 9. Stellation of cultured pituicytes is induced by β-adrenoreceptor activation and by direct activation of adenylate cyclase. Incubation of pituicyte cultures in HBSS containing isoproterenol (10 μM; Iso) or foskolin (5 μM; For) significantly increased the fraction of stellate pituicytes compared to cultures incubated in HBSS alone (Con). Incubation in the non-cyclase activating analog, 1,9 dideoxyforskolin (5 μM; DDF) or the forskolin and 1,9 DDF vehicle dimethyl sulfoxide (DMSO) had no effect on pituicytes. (✱-significantly different from HBSS alone (Con); NS - not significantly different from each other; $p < 0.05$).

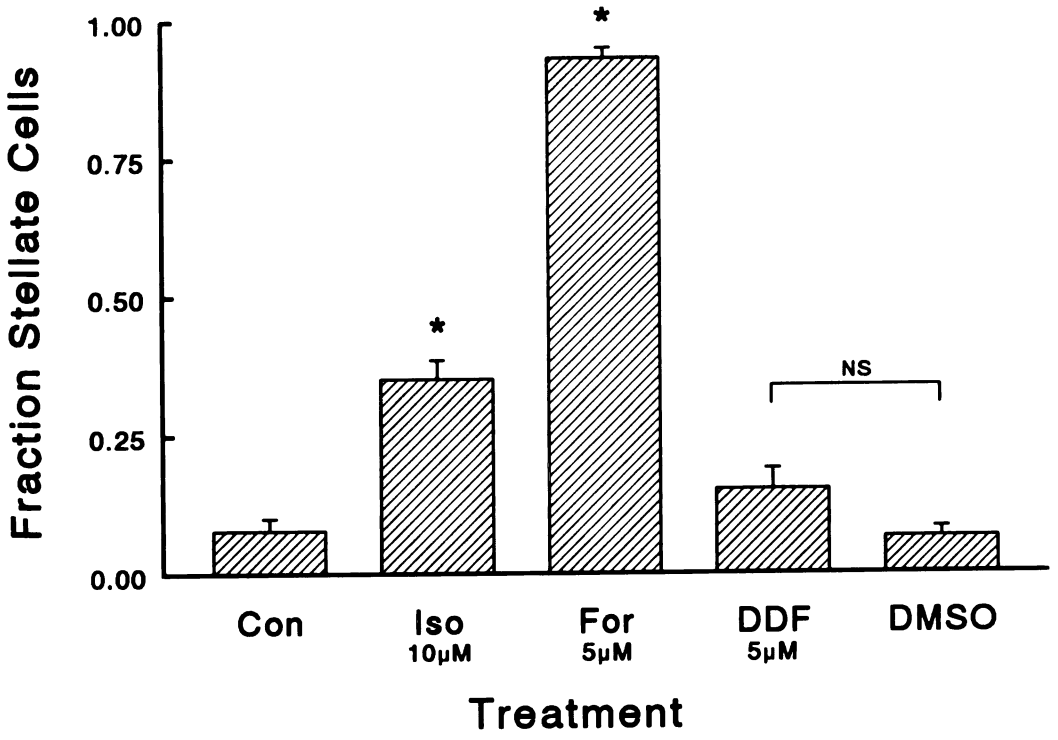

Fraction Stellate Cells
1.00
0.75
0.50
0.25
0.00
*
*
NS
Con
Iso
10μM
For
5μM
DDF
5μM
DMSO
Treatment

Figure 9

Figure 10. Forskolin induces stellation of cultured pituicytes in a dose-dependent manner. Pituicytes in culture became stellate when incubated in HBSS containing forskolin (For) compared to cultures incubated in HBSS alone (Con). The fraction of pituicytes which became stellate increased as a function of increasing forskolin concentration (For; 0.5, 1, 2.5, 5, and 10 μM). (✱ - significantly different from HBSS alone (Con); $p < 0.05$).

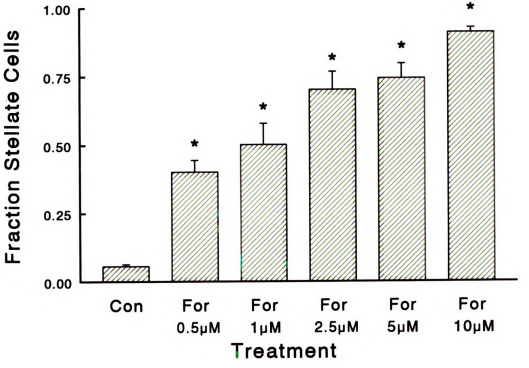

Fraction Stellate Cells
1.00
0.75
0.50
0.25
0.00
*
Con
For 0.5μM
For 1μM
For 2.5μM
For 5μM
For 10μM
Treatment

Figure 10

Figure 11. 8-bromo cyclic AMP induces stellation of cultured pituicytes. Pituicyte cultures incubated in HBSS containing forskolin (For; 5 μM) or 8-bromo cyclic AMP (8bcAMP; 150 μM) had a significantly larger fraction of stellate cells compared to cultures incubated in HBSS alone (Con). (✱ - significantly different from HBSS alone (Con); ▲ - For and 8bcAMP are significantly different from each other; $p < 0.05$).

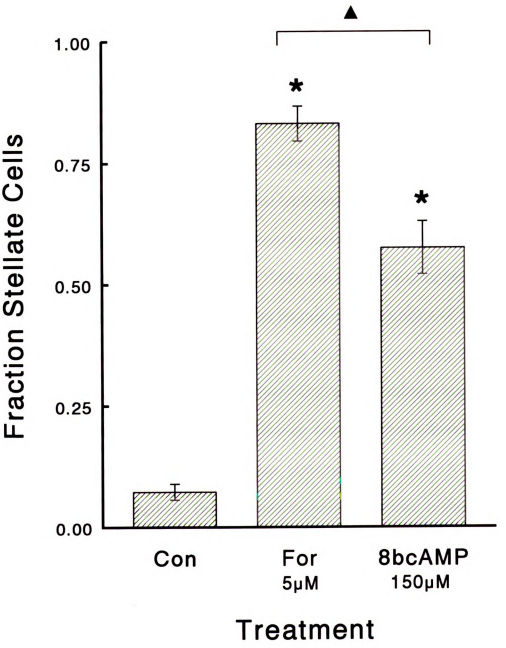

▲
1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
Con
For
5μM
8bcAMP
150μM
Treatment

Figure 11

2. IBMX and PTX Induce Stellation of Pituicytes in Culture

Intracellular cAMP concentration can be increased by reducing or inhibiting phosphodiesterase activity which is responsible for intracellular cAMP degradation. HBSS containing 3-isobutyl-1-methylxanthine (IBMX), a non-specific phosphodiesterase inhibitor (Beavo *et al.*, 1970), induced a significant fraction of cultured pituicytes to become stellate compared to cultures incubated in only HBSS (Figure 12). The effect of IBMX on pituicyte morphology indicates that increased levels of cyclic nucleotides can mediate stellation.

Pertussis toxin (PTX) selectively inhibits the adenylate cyclase linked inhibitory protein Gi by ADP-ribosylating it, and tonic inhibition of adenylate cyclase activity due to inhibitory G-protein activity can be prevented by using PTX (Bokoch *et al.*, 1983; Bokach and Gilman, 1984; Graziano and Gilman, 1987). An increase in adenylate cyclase activity can therefore be promoted with PTX if an intrinsic tonic inhibition of adenylate cyclase activity by Gi is present. As shown in Figure 13, HBSS containing PTX (100 ng/ml) induced a significant fraction of cultured pituicytes to become stellate compared to pituicytes incubated in HBSS alone. Results from this study first of all demonstrate that there is tonic Gi activity in cultured pituicytes, because inhibition of Gi by PTX induces cultured pituicytes to become stellate. This study also provides supporting evidence that increased intracellular cAMP levels can induce changes in pituicyte morphology *in vitro*.

3. Protein Kinase Inhibitors Paradoxically Induce Stellation of Cultured Pituicytes

The studies utilizing isoproterenol, forskolin, 8-bromo cAMP, IBMX, and PTX all suggest that increased intracellular cAMP concentration induces stellation. Since cAMP-

Figure 12. Isobutylmethyl xanthine (IBMX), a nonspecific phosphodiesterase inhibitor, induces stellation of cultured pituicytes. Compared to cultures incubated in HBSS alone (Con), the fraction of pituicytes that was stellate was significantly increased when pituicyte cultures were incubated in HBSS containing forskolin (For; 5 μM) or the nonspecific phosphodiesterase inhibitor IBMX (100 μM). (✱ - significantly different from HBSS alone (Con); $p < 0.05$).

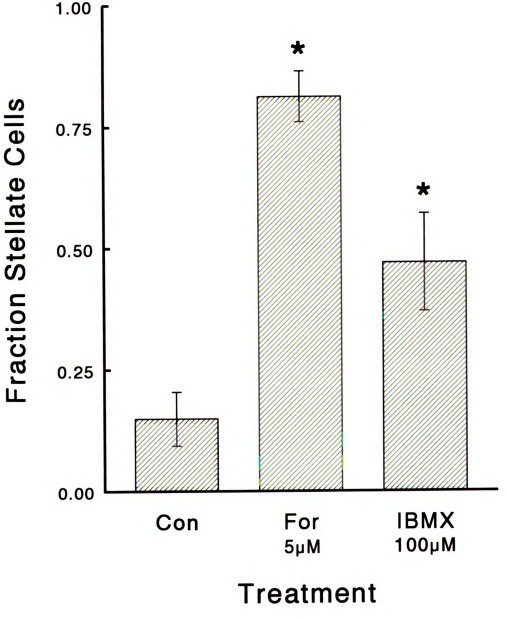

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
Con
For
5μM
IBMX
100μM
Treatment

Figure 12

Figure 13. Pertussis toxin (PTX), an inhibitor of inhibitory G-proteins, induces stellation of pituicytes in culture. A significant fraction of cultured pituicytes became stellate when incubated in HBSS containing forskolin (For; 5 μM) or in HBSS containing PTX (100 ng/ml) compared to pituicytes incubated in HBSS alone (Con). (✱ - significantly different from HBSS alone (Con); ▲ - For and PTX are significantly different from each other; $p < 0.05$).

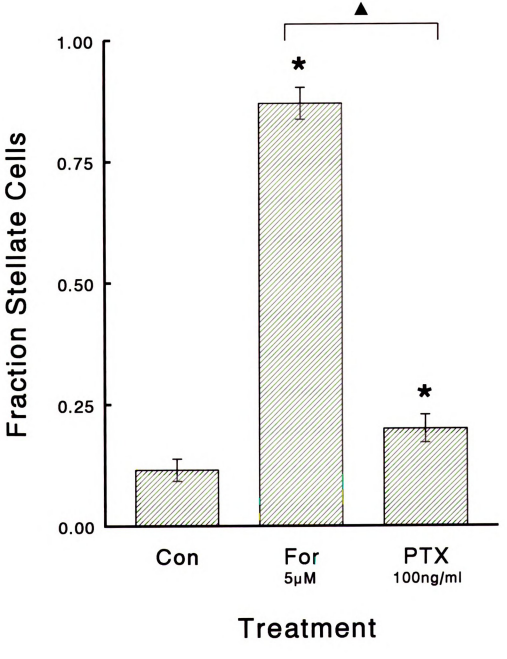

▲
1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
Con
For
5μM
PTX
100ng/ml
Treatment

Figure 13

mediated events are usually through cAMP-dependent kinase (PKA), the following studies were performed to determine if PKA mediates forskolin- or isoproterenol-induced stellation of pituicytes in culture. Three protein kinase inhibitors were used in these studies, and it was anticipated that they would inhibit cAMP-mediated stellation of pituicytes in culture by inhibiting PKA. All three PKA inhibitors paradoxically induced stellation of cultured pituicytes. H-89 ({N-[2-((3-(4-bromophenyl)-2-propenyl)amino)ethyl]-5-isoquinoline sulfonamide, HCl}) (5 μM), a selective inhibitor of PKA (Ki = 48 nM, 0.48 μM, and 31.7 μM for PKA, PKG, and PKC respectively), induced stellation of cultured pituicytes (Figure 14). In addition, rather than inhibiting forskolin-induced (1 μM) stellation as expected, H-89 actually significantly enhanced stellation induced by HBSS containing 1 μM forskolin (Figure 14). Rp-cAMPs (Rp-Cyclic 3',5'-hydrogen phophorothioate adenosine triethylamine; 10 μM) is a membrane-permeable competitive antagonist of cAMP with an apparent Ki of 8 μM, and it specifically inhibits activation of PKA I and II by cAMP (Van Hassart *et al.*, 1984). In this study, Rp-cAMPs (10 μM) induced a significant amount of stellation of pituicyte cultures compared to cultures incubated in HBSS alone (Figure 15). The fraction of stellate cells observed in response to treatment with 10 μM Rp-cAMPs in HBSS was comparable to the fraction of pituicytes which had become stellate in response to incubation of pituicytes in HBSS containing 5 μM forskolin. H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) is a selective PKA inhibitor with a Ki of 3 μM for PKA (Ki = 5.8 μM and 6 μM for PKG and PKC respectively) (Hidaka *et al.*, 1984). H-7 at concentrations of 10 and 100 μM induced a significant fraction of pituicytes in culture to become stellate compared to cultures incubated in only HBSS, and the amount of stellation induced by H-7 appeared

Figure 14. H-89, a selective cAMP-dependent protein kinase inhibitor, induces stellation of cultured pituicytes. HBSS containing forskolin (For; 1 μM), H-89 (5 μM), or both forskolin and H-89 (For, 1 μM and H-89, 5 μM) induces pituicytes in culture to become stellate compared to cultures incubated in only HBSS (Con). (✱ - significantly different from HBSS alone (Con); ▲ - significantly different from forskolin (For); $p < 0.05$).

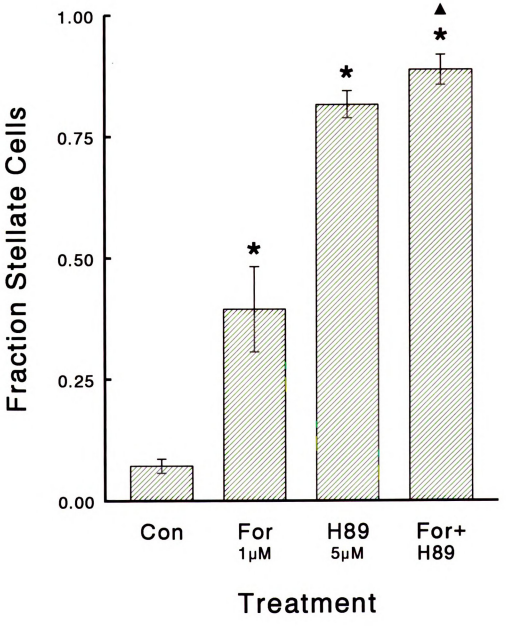

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
*
▲
Con
For
1μM
H89
5μM
For+
H89
Treatment

Figure 14

Figure 15. The cAMP-dependent protein kinase inhibitor Rp-cAMPs induces cultured pituicytes to become stellate. Cultured pituicytes incubated in HBSS containing forskolin (For; 5 μM) or in HBSS containing Rp-cAMPs (10 μM) became stellate compared to cultures incubated in HBSS alone (Con). (✱ - significantly different from HBSS alone (Con); $p < 0.05$).

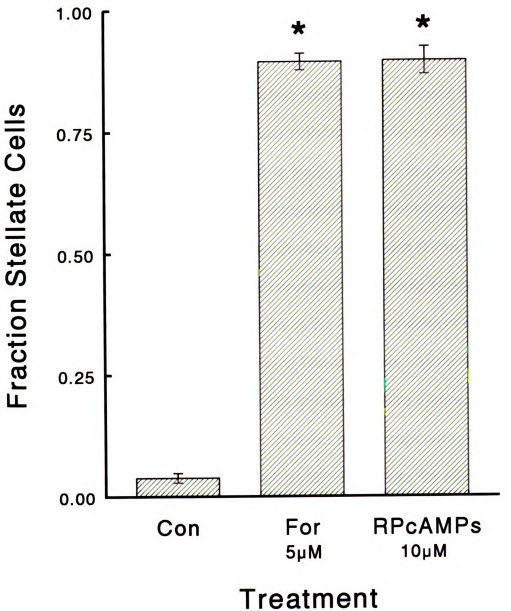

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
Con
For
5μM
RPcAMPs
10μM
Treatment

Figure 15

to be concentration dependent (Figure 16). Pituicytes were not induced to become stellate when incubated in HBSS containing 1 μM H-7. However, a significant fraction of pituicytes became stellate in response to treatment with HBSS containing 10 μM iosproterenol or with HBSS containing either 10 μM or 100 μM H-7 (Figure 16).

C. Discussion

The data from the studies presented here provide strong evidence that an increase in intracellular cAMP concentration can mediate stellation of pituicytes in culture. Adenylate cyclase produces cAMP from ATP and is highly localized in plasma membranes of pituicytes, especially where they contact neurohypophysial neurosecretory nerve fibers *in vivo* (Santolaya and Lederis, 1980). Although other studies have shown that forskolin can induce stellation of cells from glial cell lines or of astrocytes derived from neonatal rat brain, this is the first time that it has been demonstrated that direct activation of adenylate cyclase by forskolin can induce cultured pituicytes to become stellate. As the inactive forskolin analog 1,9 dideoxyforskolin, which does not activate adenylate cyclase, does not induce stellation provides further evidence that forskolin acts through adenylate cyclase, and therefore increases intracellular levels of cAMP, to mediate its effects on pituicyte morphology. The finding that forskolin induces cultured pituicytes to become stellate was expected since β-adrenoreceptor activation, which is coupled to activation of adenylate cyclase, induces pituicytes in culture to become stellate (Bicknell *et al.*, 1989 and Hatton *et al.*, 1991) and induces ultrastructural changes in isolated neurointermediate lobes (Luckman and Bicknell, 1990; Smithson *et al.*, 1990).

Figure 16. H-7, a selective inhibitor of cAMP-dependent protein kinase, induces stellation of cultured pituicytes. The fraction of pituicytes that was stellate was significantly increased when the cultures were incubated in HBSS supplemented with either the β-adrenergic agonist isoproterenol (Iso; 10 μM) or the cAMP-dependent protein kinase inhibitor H-7 (10 and 100 μM) compared to cultures incubated in HBSS alone (Con). (✱ - significantly different from HBSS alone (Con); ▲ - significantly different from isoproterenol (Iso); NS - Iso (10 μM) and H-7 (1 μM) are not significantly different from each other; $p < 0.05$).

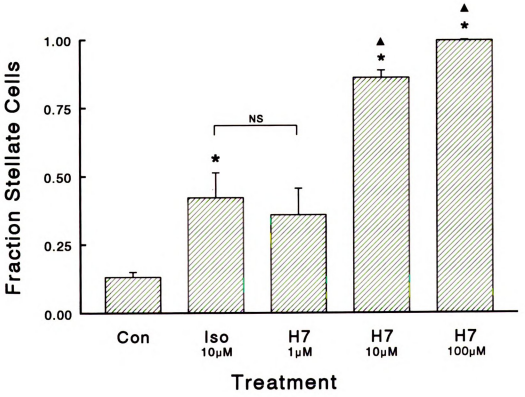

Fraction Stellate Cells
1.00
0.75
0.50
0.25
0.00
NS
Con
Iso
10μM
H7
1μM
H7
10μM
H7
100μM
Treatment

Figure 16

The original proposal that the diverse actions of cAMP on eukaryotic physiology are all mediated through cAMP-dependent protein kinases (Kuo and Greengard, 1969) is the principle working hypothesis for the mechanism of action of cAMP (Rosen, 1977). Many cAMP analogs can efficiently activate more than one cAMP-dependent protein kinase (Meyer and Miller, 1974; Miller, 1977), although many studies on the activation of protein kinases by cAMP analogs were completed before it became generally recognized that there are two isozymic forms of cAMP-dependent protein kinase, termed type I (PK I) and type II (PK II) (Corbin *et al.*, 1975a,b; Hoffmann *et al.*, 1975). Since PK I and PK II isozymes differ principally in their regulatory units (Hoffmann *et al.*, 1975; Fleischer *et al.*, 1976), some analogs of cAMP are more potent activators of PK I than PK II, and vice versa. The data presented here demonstrate that the cAMP analog 8-bromo cAMP can induce stellation of cultured pituicytes, and further indicate that increased intracellular cAMP levels can cause stellation of pituicytes in culture. The fact that 8-bromo cAMP, which induces stellation of cultured pituicytes, is selective for site 1 on PK I and PK II, just as cAMP is (Beebe *et al.*, 1988), implicates PKA as a mediator in the biochemical pathway which results in stellation of pituicytes.

Pertussis toxin, which is an inhibitor of inhibitory G-proteins, significantly increased the fraction of cultured pituicytes which became stellate compared to pituicyte cultures incubated in HBSS alone. Since there are no endogenous activators of Gi in HBSS, this finding indicates that there is tonic inhibitory G-protein activity present in pituicytes. Tonic Gi-protein activity may function in pituicytes to inhibit adenylate cyclase and prevent increased levels of cAMP which are necessary for stellation, and therefore contribute to the

maintenance of a non-stellate morphology. That inhibition of Gi by pertussis toxin, and therefore disinhibition of adenylate cyclase, causes stellation of cultured pituicytes provides additional evidence that an increase in intracellular cAMP concentration can mediate stellation of pituicytes *in vitro*.

There has recently been much interest in the ability of phosphodiesterases (PDEs) to modulate second messenger systems. There are currently at least seven different gene families of PDEs and more than 30 different isozymes of phosphodiesterases which have been recognized (Beavo *et al.*, 1994). Many cyclic nucleotide-specific PDEs serve specific functions in cells in which they are expressed, and it has become clear that PDEs can play a crucial role in mediating cross-talk between second-messenger pathways, since individual PDE enzymes can be positively and negatively regulated by second messengers (Beavo *et al.*, 1994). 3-isobutylmethyl-1-xanthine (IBMX) is a xanthine derivative that decreases phosphodiesterase activity in cells (Beavo *et al.*, 1970). Therefore, if there is concurrent cyclic nucleotide production by cyclases and PDE-mediated cyclic nucleotide degradation, IBMX can inhibit phosphodiesterase activity and consequently increase intracellular cyclic nucleotide concentrations. Data presented here demonstrate that IBMX can induce stellation of cultured pituicytes, revealing that there must be both tonic cyclic nucleotide production by cyclases and degradation of cyclic nucleotides by active phosphodiesterases in cultured pituicytes. These results further indicate that increased intracellular cyclic nucleotide concentration causes stellation of cultured pituicytes.

Cyclic AMP-dependent protein kinase (PKA) is an enzymatic protein present in all mammalian tissues and typically has a cellular level of 0.2-2 μM (Shabb and Corbin, 1992).

In the absence of cAMP, PKA is inactive and is composed of two regulatory subunits and two catalytic subunits. When two molecules of cAMP bind to each regulatory subunit, the catalytic subunits dissociate from the regulatory subunits and the catalytic subunits become active. Active PKA transfers the γ-phosphate of ATP to serine or threonine residues on many cellular enzymes, and it has been shown to phosphorylate a number of proteins and therefore regulate a number of cellular pathways (see Francis and Corbin, 1994). cAMP-dependent protein kinase can phosphorylate cyclic nucleotide phosphodiesterases to regulate intracellular cyclic nucleotide levels, phosphorylate other kinases, such as myosin light kinase which is involved in regulation of the cytoskeleton, and even phosphorylate its own regulatory and catalytic subunits (see Shacter *et al.*, 1988). PKA has also been shown to phosphorylate a variety of cytoskeletal proteins such as actin, filamin, MAP (microtubule associated protein) 2, and neurofilaments (see Shacter *et al.*, 1988). One study demonstrated that phosphorylation by PKA reversibly inhibits the bundling of actin filaments, further implicating PKA as a regulator of the cytoskeleton (Stossel, 1989).

A reasonable assumption based on the initial findings from pituicyte experiments presented here would be that increased cAMP concentration in cultured pituicytes leads to activation of cAMP-dependent protein kinase which in turn either directly or indirectly alters the phosphorylation status of cytoskeletal proteins which therefore results in a stellate morphology. However, the observation that the protein kinase A inhibitors H-89, Rp-cAMPs, and H-7 by themselves induce stellation of cultured pituicytes does not support this hypothesis and therefore makes interpretation of the data difficult. It may be that the protein kinase inhibitors are having effects not mediated through protein kinase A. However, Rp-

cAMPs competes with cAMP for the regulatory subunit of PKA (Van Haastert *et al.*, 1984), while H-89 inhibits PKA by competing for the ATP binding site on the kinase (Chijiwa *et al.*, 1990). The fact that Rp-cAMPs and H-89, for example, have the same effect on pituicyte morphology, but are structurally different from each other and inhibit PKA by two different mechanisms, makes nonspecific actions seem unlikely.

The effects of cAMP-inducing agents and PKA inhibitors on pituicyte morphology appear to be paradoxical, but it is possible to explain this discrepancy with hypothetical biochemical mechanisms of action. Most effects of cAMP in mammals are mediated via cAMP-dependent protein kinase (PKA) with subsequent phosphorylation of proteins. It is possible that in cultured pituicytes the non-stellate morphology is maintained during basal, non-zero, levels of PKA activity. Therefore, when PKA activity is increased by increasing levels of intracellular cAMP or PKA activity is decreased by inhibition via a PKA inhibitor, PKA deviates from its basal level of activity and a stellate morphology may be obtained.

It is also possible that cAMP may be mediating its effects on pituicyte morphology through a biochemical mechanism which does not involve PKA. cAMP has been shown to regulate ion channels and to bind cGMP-binding sites of PKG to exert its effects, such as that observed in the olfactory system (Ludwig *et al.*, 1990). It has also been demonstrated that either cAMP or cGMP can bind to protein kinase G (PKG) and mediate vascular smooth muscle relaxation (Hardman, 1984; Murad, 1986; Lincoln, 1989). Whether cAMP is modulating ion channels or regulating the activity of PKG in cultured pituicytes is currently unknown.

There are two major mammalian PKA regulatory subunit isoforms, each with

subclasses (RIα, RIβ, RIIα, and RIIβ), and three major forms of the mammalian PKA catalytic subunit (Cα,Cβ, and Cγ). Combinations of the different regulatory and catalytic subunits offer 24 different holoenzymes which could be present in mammalian tissues (Francis and Corbin, 1992). *In vitro*, differences in the affinity of cAMP for the different regulatory subunits has been observed, and dissociation of the regulatory and catalytic subunits may occur at different rates (Francis and Corbin, 1994). In eukaryotes, the regulatory subunit of PKA has been defined in terms of its function to inhibit the catalytic subunit in the holoenzyme state, where binding of cAMP to the regulatory subunit greatly reduces the affinity of the regulatory subunit for the catalytic subunit, resulting in dissociation of the regulatory subunit from catalytic subunit and activation of the free catalytic subunit (Flockhart and Corbin, 1982).

Only recently have researchers demonstrated that it is possible for the cAMP-bound regulatory subunits to exert an effect independent of activation of of the catalytic subunit (Ludwig *et al.*, 1990). A several fold increase of the regulatory subunit (either RI or RII) over the catalytic subunit can be generated in certain tissues *in vivo*, or in cultured cells, treated with dBcAMP (Lohmann and Walter, 1984). The first RII binding protein other than the catalytic subunit was identified as MAP (microtubule associated protein) 2. Association of PKA with cytoskeletal components such as MAP has been shown to be mediated via the regulatory subunit, and it has been estimated that up to one third of total cytosolic PKA in brain is bound via RII to MAP 2 (Theurkauf and Vallee, 1982). cAMP promotes the dissociation of the catalytic subunit from the regulatory subunit, but not the dissociation of the regulatory subunit from substrates such as MAP (Corbin *et al.*, 1977; Theurkauf and

Vallee, 1982). Three major brain proteins (68, 80, and 300 KDa) have been copurified with RII (Lahman *et al.*, 1988). The 300 KDa protein has been shown to be MAP, and the RII binding protein of 68 KDa was shown to copurify with MAP and PKA and has been suggested to be a cytoskeletal protein (Vallee *et al.*, 1981). As the regulatory subunit has been shown to inhibit some phosphatase activities, it is possible that inhibition of the regulatory subunit via Rp-cAMPs for example, results in increased phosphatase activity of particular proteins which leads to stellation. If increased cAMP activates protein kinase A and induces stellation via phosphorylation of a protein such as myosin light chain kinase, this would indicate that cAMP and PKA inhibitors could induce stellation of cultured pituicytes via two distinct pathways.

Data from the pituicyte experiments presented here demonstrate that increased intracellular cAMP concentration can induce stellation of these cells. Tonic Gi-protein and phosphodiesterase activities appear to be present in pituicytes, possibly to maintain the cells in a non-stellate morphology until induced to become stellate. Experiments with the cAMP analog 8-bromo cAMP indicate that PKA is involved in mediating stellation. Although effects of the PKA inhibitors on pituicyte morphology appear to be paradoxical, there are hypothetical mechanisms which may explain their mode of action.

III. cGMP Mediates Stellation of Cultured Pituicytes

A. Introduction and Experimental Rationale

Nitric oxide (NO) is a biological molecule with important functions in the immune, cardiovascular, and nervous systems. Nitric oxide has been demonstrated to activate soluble guanylate cyclase and elevate cGMP levels in the brain (Katsuki *et al.*, 1977; Miki *et al.*, 1977). Cyclic GMP can activate cGMP-dependent serine/threonine kinase, cGMP-dependent cation channels (Light *et al.*, 1989; Fesenko *et al.*, 1991) and cGMP-dependent phosphodiesterases (Beavo and Reifsnyder, 1990), but unfortunately little is known about the functions of cGMP in the brain. Nitric oxide is synthesized by nitric oxide synthase (NOS), which is an enzyme that produces NO and L-citrulline by catalyzing the oxidation of one of the terminal guanidino nitrogens of L-arginine (Palmer *et al.*, 1988). Several isoforms of NOS have been purified and cloned, and they represent a novel family of proteins that contain domains for both heme and cytochrome P450 reductase (Stuehr and Ikeda-Saito, 1992; White and Marletta, 1992).

Inducible, neuronal, and endothelial NOS are three NOS isoforms which are derived from separate genes and regulated by diverse signal transduction pathways. Inducible NOS (iNOS) is found largely in the immune system and in smooth muscle and liver cells (Murphy *et al.*, 1993). It has been shown that astrocytes exhibit both constitutive and inducible NOS activity under various conditions and that activated microglia express inducible NOS (Murphy *et al.*, 1993). The enzymatic activity of iNOS requires stimulation by a cytokine and requires new protein synthesis but is Ca^{+2} and calmodulin independent (Iyengar *et al.*, 1987). Constitutive forms of NOS (cNOS) have been localized to neurons (nNOS) in the

central and peripheral nervous systems (cytosolic) and to the endothelium (eNOS) (membrane bound). These constitutive forms of NOS can rapidly increase their enzymatic activity, which is dependent on Ca^{+2} and calmodulin (Palmer *et al.*, 1988). Thus in cells in which cNOS is present, NO may be synthesized independently of new protein synthesis.

Neuronal NOS (Bredt *et al.*, 1990) and endothelial NOS (Dinerman *et al.*, 1994) have been localized to discrete cell populations in several regions of the brain such as the cerebellum, olfactory bulb, and the hippocampus. Neuronal NOS has been demonstrated in the SON and PVN magnocellular neurons (Bredt *et al.*, 1990; Huang *et al.*, 1993; and Dawson *et al.*, 1994) and cell bodies (Bredt *et al.*, 1990; Vincent and Kimura, 1992; Huang *et al.*, 1993). NADPH (β-nicotimamide adenine dinucleotide phosphate, reduced) diaphorase staining is an enzymatic process whereby neuronal NOS (Dawson *et al.*, 1991; Hope *et al.*, 1991) reduces tetrazolium dyes to a blue formazan precipitate in the presence of NADPH but not in the presence of NADH (Thomas and Pearse, 1961, 1964). NAPDH-diaphorase staining is indicative of NOS localization, and NADPH-diaphorase appears to be a form of NOS itself (Hope *et al.*, 1991). Although staining for NADPH-diaphorase has been demonstrated in the neurohypophysis, the intermediate and anterior lobes of the pituitary do not stain for NADPH-diaphorase (Sagar and Ferriero, 1987).

Several recent studies have implicated nitric oxide as a regulatory molecule in the HNS. Dehydration and salt loading are associated with increased staining for NOS in the SON (Pow, 1992; Villar *et al.*, 1994; Calka *et al.*, 1994), PVN (Villar *et al.*, 1994), and neurohypophysis (Sagar and Ferriero, 1987). NOS mRNA has been shown to increase in the PVN in response to immobilization stress (Calzà *et al.*, 1993) and lactation (Ceccatelli and

Eriksson, 1993), and in both the PVN and SON of rats after salt loading (2% NaCl drinking) (Villar *et al.*, 1994). Beagley and Cobbett (1996) have demonstrated that rats given an intraperiotoneal injection of 1.5 M NaCl along with the NOS inhibitor L-NAME have a significantly reduced amount of axon terminal apposition with the basal lamina in the neurohypophysis compared to rats injected with only NaCl. This study indicates that nitric oxide may play a role in regulating ultrastructural relations in the neurohypophysis of osmotically-induced rats, and it may be predicted that nitric oxide would induce stellation of cultured pituicytes. However, since the L-NAME was not administered directly to the neurohypophysis, their study did not provide unequivical evidence that NO exerts its effects on neurohypophysial ultrastructure by acting within the neurohypophysis. Intracerebroventricular injection of the NOS inhibitors L-NMMA and L-NAME have been shown to enhance plasma levels of oxytocin after 24 hr of water deprivation (Summy-Long *et al.*, 1993). This study suggests that nitric oxide may function to regulate hormone secretion from the neurohypophysis, and therefore nitric oxide may have effects other than altering ultrastructural plasticity in the neurohypophysis. These studies indicate that nitric oxide may function to regulate peptide hormone secretion from the neurohypophysis into the systemic circulation by altering ultrastructural relations in the neurohypophyis or by affecting the magocellular neurons directly.

Soluble guanylate cyclase, which is an enzyme located in the cytoplasm of some cells, is activated by nitric oxide, and it can be activated by nitric oxide donors such as sodium nitroprusside (Waldman and Murad, 1987) and SIN-1 (Bowen and Haslam, 1991; Jones *et al.*, 1994). Nitric oxide has been shown to activate soluble guanylate cyclase in

the central nervous system (Katsuki *et al.*, 1977, Garthwaite *et al.*, 1988), and soluble guanylate cyclase is distributed throughout the brain in subpopulations of astrocytes and in a few neuronal cell bodies (De Vente and Steinbush, 1992). It is possible that neurohypophysial pituicytes could contain soluble guanylate cyclase and serve as a target for nitric oxide produced by NOS in the magnocellular neurons. Therefore the effects of nitric oxide and activation of soluble guanylate cyclase on pituicyte morphology have been investigated in the following studies presented here.

Atrial natriuretic peptide (ANP) is a biologically active peptide involved in regulation of renal, adrenal, and cardiovascular functions (Bahr *et al.*, 1993; O'Donnell *et al.*, 1988). ANP binds to ANP cell surface receptors which have intrinsic guanylate cyclase activity, and activation of this particulate guanylate cyclase results in production of cGMP from GTP (Garbers, 1989; Hamet *et al.*, 1984; Waldman *et al.*, 1984). In some cases increased cGMP results in inhibition of adenylate cyclase activity (Anand-Srivastave *et al.*, 1984).

In the hypothalamus, ANP-immunoreactivity with oxytocin has been demonstrated in a small percentage of neurons (Jirikowski *et al.*, 1986). The effects of ANP on vasopressin secretion from the neurhypophysial nerve terminals is controversial, as ANP has been shown to both supress (Montes and Johnson, 1989) and enhance (Januszewicz *et al.*, 1985) vasopressin secretion *in vivo*. Endogenous ANP and ANP receptors are localized in the rat neurohypophysis (Gutkowska *et al.*, 1987), and ANP receptors have been shown to be localized on pituicytes (Luckman and Bicknell, 1991). Although one study has indicated that ANP has no effect on basal or electrically stimulated release of oxytocin or vasopressin secretion from isolated neural lobes or neurointermediate lobes *in vitro* (Luckman and

Bicknell, 1991), other investigators have shown that ANP can reduce stimulated release of neurohypophysial hormones *in vitro* from hypothalamo-neurohypophysial explants (Crandall and Gregg, 1986) and from isolated neurointermediate lobes (Poole *et al.*, 1987).

In contrast to nitric oxide which activates soluble guanylate cyclase, ANP activates particulate guanylate cyclase to increase intracellular cGMP concentration. Particulate guanylate cyclase has been shown to be localized in both pituicytes and in neurosecretory terminals in the neurohypophysis by localization of a lead precipitate which is formed at the site of production of inorganic pyrophosphate by guanylate cyclase from its substrate (Rambotti *et al.*, 1994). The presence of atrial natriuretic peptide in the neurohypophysis and ANP receptors on pituicytes indicates a possible role for particulate guanylate cyclase to serve some regulatory function. Whether or not atrial natriuretic peptide can affect pituicyte morphology has therefore been addressed in the following studies.

B. Results

1. Nitric Oxide Donors and the Cyclic cGMP Analog 8-bromo cGMP Mediate Stellation of Pituicytes in Culture

The nitric oxide donors sodium nitroprusside (SNP) and 3-morpholinosydnonimine (SIN-1) were used to investigate whether nitric oxide could alter pituicyte morphology *in vitro*. Pituicyte cultures incubated in HBSS containing either SNP (10 μM) or SIN-1 (10 μM) had a significantly larger fraction of stellate cells compared to cultures incubated in only HBSS (Figure 17). This study demonstrates that nitric oxide donors can induce stellation of pituicytes in culture.

Figure 17. Nitric oxide donors (SNP and SIN-1) mimick β-adrenoreceptor mediated stellation of cultured pituicytes. Compared to cultures incubated in HBSS alone (Con), the fraction of pituicytes that was stellate was significantly increased when cultures were incubated in HBSS supplemented with isoproterenol (Iso; 20 μM) or in HBSS supplemented with the nitric oxide donors sodium nitroprusside (SNP; 10 μM) or SIN-1 (SIN; 10 μM). (✱ - significantly different from HBSS alone (Con); $p < 0.05$).

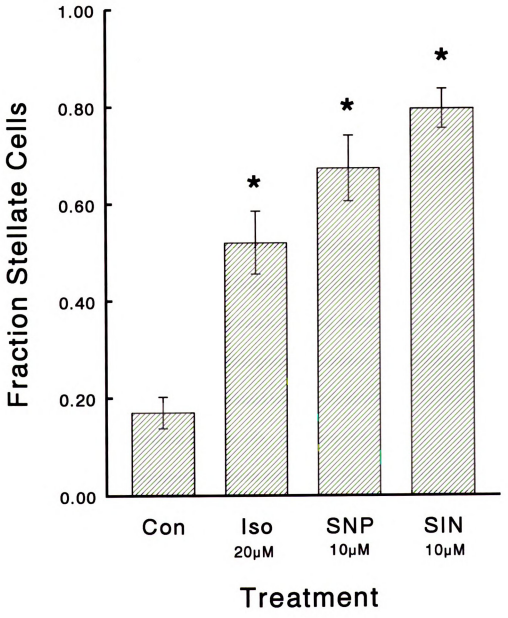

Fraction Stellate Cells
1.00
0.80
0.60
0.40
0.20
0.00
*
*
*
Con
Iso
20μM
SNP
10μM
SIN
10μM
Treatment

Figure 17

Since nitric oxide donors can activate soluble guanylate cyclase and are presumed to increase intracellular cGMP concentration, the studies presented here have investigated whether elevated levels of intracellular cGMP, by incubating pituicyte cultures in HBSS containing the membrane permeable analog 8-bromo cGMP (8-bromo guanosine 3',5'-cyclic monophosphate) can induce stellation. A significant fraction of pituicytes was induced to become stellate when cultures were incubated in HBSS supplemented with 8-bromo cGMP (100 μM) compared to cultures incubated in HBSS alone (Figure 18). Results from this study indicate that increased intracellular cGMP concentration can mediate stellation of cultured pituicytes.

The soluble guanylate cyclase inhibitors methylene blue (Mayer *et al.*, 1993) and LY 83,583 (6-anilo-5,8-quinolinedione) (Mülsch *et al.*, 1989; Brandt and Conrad, 1991) were used to confirm that sodium nitroprusside-induced stellation of cultured pituicytes was indeed mediated by activation of soluble guanylate cyclase. The stellation response induced by SNP (25 μM) was significantly reduced when methylene blue (25 μM) was also included in the experimental medium (Figure 19). Inclusion of LY-83,583 (10 μM) in the SNP-containing HBSS experimental solution completely prevented SNP-induced stellation of pituicytes in culture (Figure 20). These results provide supporting evidence that SNP mediates stellation by release of nitric oxide which activates soluble guanylate cyclase. It is interesting to note that treatment of pituicyte cultures with HBSS containing 10 μM LY 83,583 resulted in a significantly smaller fraction of stellate cells compared to cultures incubated in HBSS alone (Figure 20) and that LY-83,583 also inhibits forskolin-induced

Figure 18. Stellation of cultured pituicytes is induced by 8-bromo cyclic GMP. Compared to pituicyte cultures incubated in HBSS alone (Con), cultures incubated in HBSS containing sodium nitroprusside (SNP) or the membrane permeable cyclic GMP analog 8-bromo cyclic GMP (8bcGMP) had a higher fraction of stellate cells. (✱ - significantly different from control cultures (Con); $p < 0.05$).

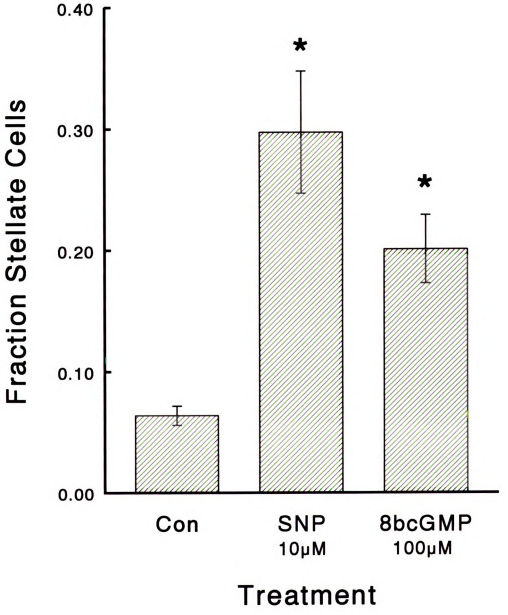

0.40
0.30
0.20
0.10
0.00
Fraction Stellate Cells
*
*
Con
SNP
10μM
8bcGMP
100μM
Treatment

Figure 18

Figure 19. The soluble guanylate cyclase inhibitor methylene blue attenuates sodium nitroprusside-induced stellation of pituicytes in culture. Compared to pituicyte cultures incubated in HBSS alone (Con), the fraction of stellate cells was significantly increased when cultures were incubated in HBSS supplemented with sodium nitroprusside (SNP; 25 μM) or in HBSS supplemented with 8-bromo cyclic GMP (8-br cGMP; 200 μM). Stellation of pituicyte cultures was not induced when cells were incubated in HBSS supplemented with only methylene blue (MB; 25 μM). Sodium nitroprusside-induced stellation was significantly attenuated when pituicyte cultures were incubated in HBSS supplemented with both sodium nitroprusside and methylene blue (SNP+MB). 8-bromo cyclic GMP induced stellation of cultured pituicytes was not significantly attenuated when cultures were incubated in HBSS supplemented with 8-bromo cyclic GMP and methylene blue (8-br cGMP+MB). (✱ - significantly different from Con; ▲ - significantly different from SNP; NS - not significntly different; $p < 0.05$).

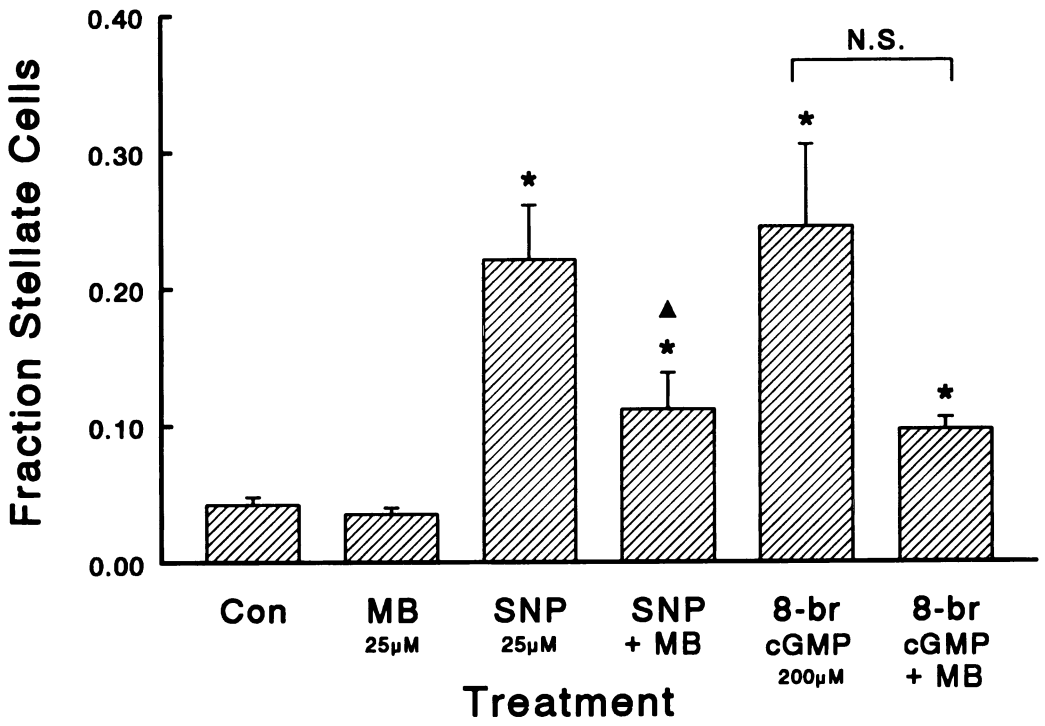

Fraction Stellate Cells
0.40
0.30
0.20
0.10
0.00
N.S.
*
*
▲
*
*
Con
MB
25μM
SNP
25μM
SNP
+ MB
8-br
cGMP
200μM
8-br
cGMP
+ MB
Treatment

Figure 19

Figure 20. The soluble guanylate cyclase inhibitor LY-83,583 inhibits sodium nitroprusside-induced stellation of cultured pituicytes. Pituicyte cultures incubated in HBSS containing sodium nitroprusside (SNP; 10 μM) or SIN-1 (SIN; 10 μM) had a significantly larger fraction of stellate pituicytes compared to cultures incubated in HBSS alone (Con). Cultures incubated in HBSS containing both LY-83583 (10 μM) and sodium nitroprusside (SNP+LY) had a significantly smaller fraction of stellate pituicytes compared to cultures incubated in HBSS containing only sodium nitroprusside (SNP). Note that compared to cultures incubated in HBSS alone (Con), the fraction of pituicytes that was stellate was significantly decreased when pituicyte cultures were incubated in HBSS containing LY-83583 (LY) only. (✱ - significantly different from HBSS alone (Con); ▲ - significantly different from SNP; NS - not significantly different; $p < 0.05$).

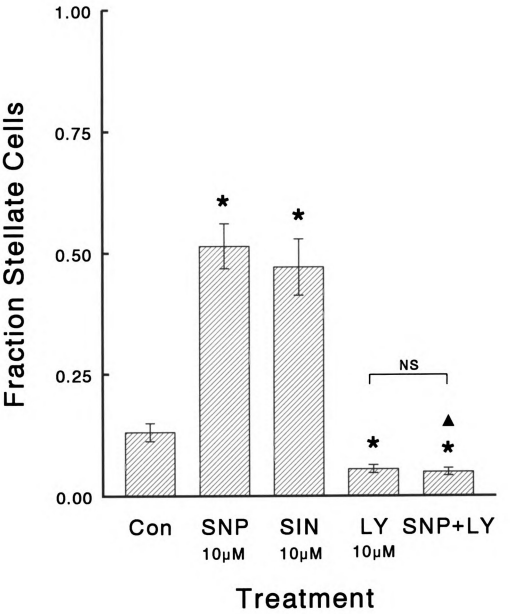

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
NS
*
▲
*
Con
SNP
10μM
SIN
10μM
LY
10μM
SNP+LY
Treatment

Figure 20

Figure 21. LY-83,583 inhibits forskolin-induced stellation of cultured pituicytes. Incubation of pituicyte cultures in HBSS containing forskolin (For) induced a significant fraction of pituicytes to become stellate compared to controls (Con). Incubation of cultures in HBSS containing both forskolin and LY-83583 (For+LY) resulted in a significantly smaller fraction of stellate pituicytes compared to cultures incubated in HBSS containing forskolin (For) alone. Also, compared to cultures incubated in HBSS alone (Con), incubation of cultures in HBSS containing LY-83583 (LY) or in HBSS supplemented with both LY-83583 and forskolin (For+LY) resulted in a significantly smaller fraction of stellate pituicytes. (✱ - significantly different from HBSS alone (Con); ▲ - significantly different from forskolin; $p < 0.05$).

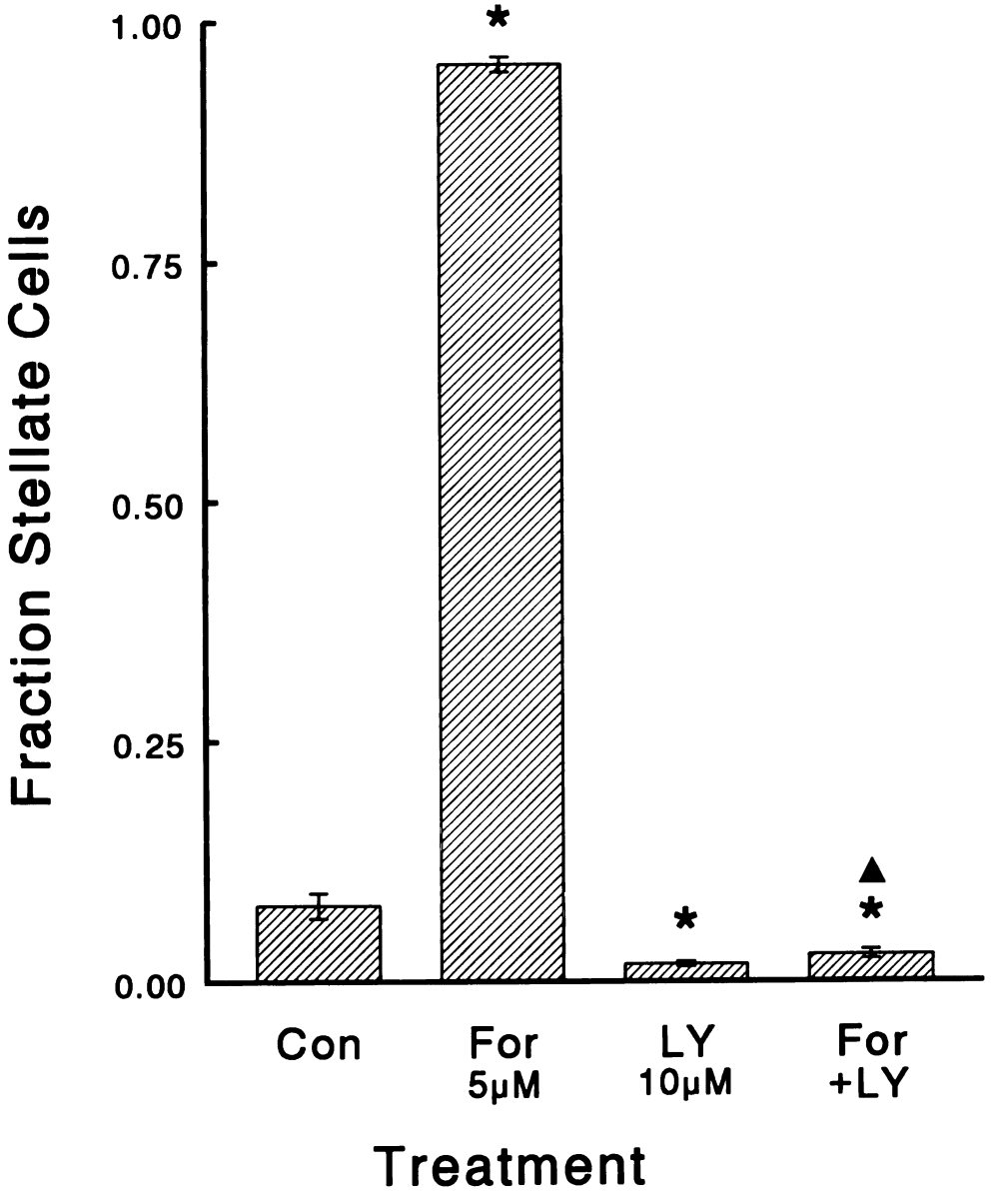

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
▲
*
Con
For
5μM
LY
10μM
For
+LY
Treatment

Figure 21

stellation of pituicytes in culture (Figure 21).

2. cGMP-Dependent Phosphodiesterase Inhibitors Induce Stellation of Cultured Pituicytes

Incubation of pituicyte cultures in HBSS supplemented with 5 μM MY-5445 (1-(3-chlorophenylamino)-4-phenylphthalazine), a cGMP-specific PDE (PDE 5) inhibitor (Akamatsu *et al.*, 1993) resulted in a significantly larger fraction of stellate pituicytes compared to pituicytes incubated in HBSS alone (Figure 22). MY-5445 was also shown to enhance SNP-induced stellation since incubation of pituicyte cultures in HBSS containing both SNP and MY-5445 had a significantly larger fraction of stellate cells compared to cultures incubated in HBSS containing only SNP. Pituicytes incubated in HBSS supplemented with 25 μM milrinone (1,6-Dihydro-2-methyl-6-oxo-3,4-bipyridine), an inhibitor of cGMP-inhibited PDE (PDE 3) (Harrison *et al.*, 1986), were also induced to become stellate compared to cultures incubated in only HBSS (Figure 23). Results from these studies indicate that stellation of cultured pituicytes can be induced by increased intracellular cGMP concentration via inhibition of cGMP-specific phosphodiesterase (PDE 5), which hydrolyzes only cGMP, or by increased intracellular cAMP concentration via inhibition of cGMP-inhibited phosphodiesterase (PDE 3), which hydrolysis only cAMP. These data suggest that either increased intracellular concentration of either cAMP or cGMP can mediate stellation of cultured pituicytes. A suggested biochemical pathway of how MY-5445, milrinone, and LY-83,583 may affect pituicyte morphology is shown in Figure 25.

Figure 22. MY-5445, a cGMP-specific phosphodiesterase inhibitor, induces stellation of pituicytes in culture and enhances sodium nitroprusside-induced stellation. Pituicyte cultures incubated in HBSS containing sodium nitroprusside (SNP; 10 μM) or in HBSS containing the cGMP-specific phosphodiesterase inhibitor MY-5445 had a significantly larger fraction of stellate cells compared to cultures incubated in HBSS alone (Con). Incubation of pituicytes in HBSS supplemented with both sodium nitroprusside and MY-5445 (SNP+MY) resulted in a larger fraction of stellate pituicytes compared to cultures incubated in HBSS (Con), sodium nitroprusside (SNP; 10 μM) or MY-5445 (MY; 5 μM) alone. (✱ - significantly different from Con; ▲ - significantly different from SNP; ■ - significantly different from MY; $p < 0.05$).

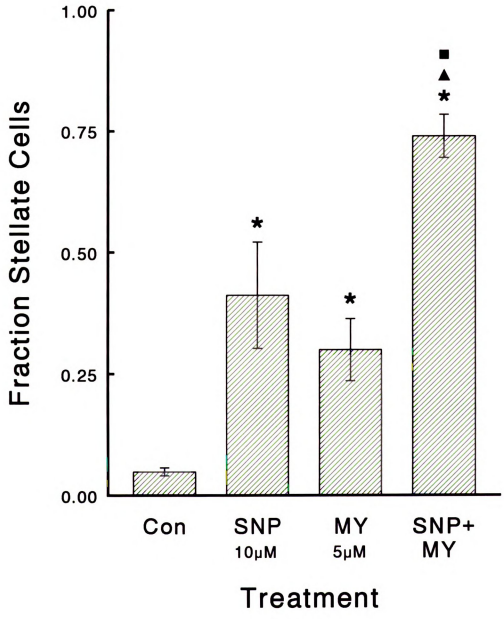

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
■
▲
*
Con
SNP
10μM
MY
5μM
SNP+
MY
Treatment

Figure 22

Figure 23. Milrinone, a cGMP inhibited phosphodiesterase inhibitor, induces stellation of cultured pituicytes. Incubation of pituicyte cultures in HBSS containing forskolin (For; 5 μM) or in HBSS containing milrinone, an inhibitor of cGMP-inhibited phosphodiesterase, (Mil; 25 μM) induced a significant fraction of pituicytes to become stellate compared to cultures incubated in HBSS alone (Con). (✱ - significantly different from HBSS alone (Con); $p < 0.05$).

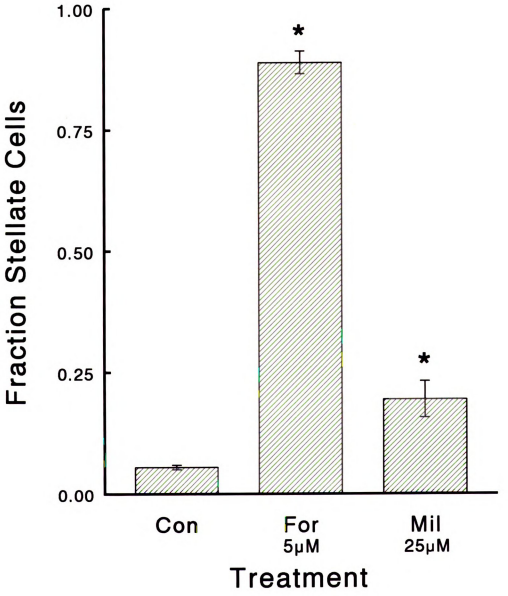

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
Con
For
5μM
Mil
25μM
Treatment

Figure 23

Figure 24. Atrial natriuretic peptide induces cultured pituicytes to become stellate. The fraction of pituicytes which was stellate was significantly increased when cultures were incubated in HBSS containing forskolin (For; 5 μM) or in HBSS supplemented with atrial natriuretic peptide (ANP; 1 μM) compared to cultures incubated in HBSS alone (Con). (✱ - significantly different from Con; $p < 0.05$).

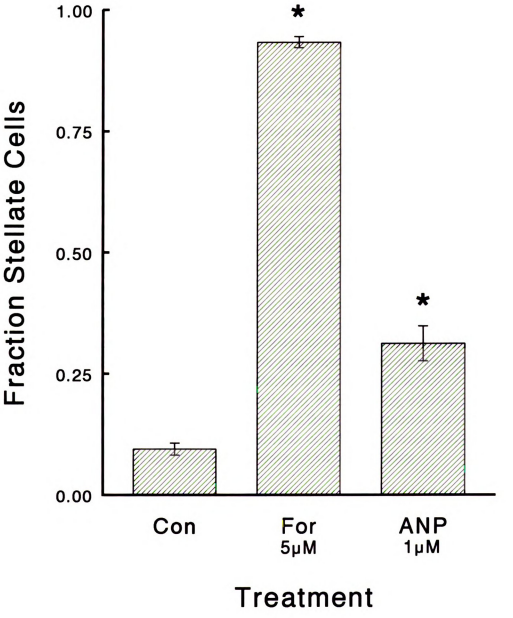

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
Con
For
5μM
ANP
1μM
Treatment

Figure 24

Figure 25. Suggested biochemical pathways mediating the effects of nitric oxide, ANP, MY-5445, milrinone, and LY-83,583 on pituicyte morphology *in vitro*. Nitric oxide appears to activate soluble guanylate cyclase, whereas atrial natriuretic peptide most likely activates a membrane bound ANP receptor coupled to an intrinsic particulate guanylate cyclase, to increase intracellular cGMP levels and induce stellation of pituicytes in culture. Whether cGMP is activating protein kinase G, affecting intracellular cAMP levels, or acting through some other mechanism to mediate stellation is unclear. Milrinone, which inhibits PDE-3 (which selectively hydrolyzes cAMP) can inhibit the degradation of cAMP to 5'AMP. If cAMP is already being produced, milrinone could therefore increase intracellular cAMP concentration to alter pituicyte morphology. MY-5445, which inhibits PDE-5 (which selectively hydrolyzes cGMP) can inhibit cGMP degradation and therefore increase intracellular cGMP concentration if cGMP is already being produced. It is unclear whether this increase in intracellular cGMP directly affects pituicyte morphology or whether cGMP inhibits PDE-3 to increase intracellular cAMP concentration and therefore indirectly alter pituicyte morphology. LY-83,583 inhibits soluble guanylate cyclase and can inhibit both sodium nitroprusside- and forskolin-induced stellation of cultured pituicytes. LY-83,583 likely inhibits sodium nitroprusside-induced stellation by inhibiting soluble guanylate cyclase and therefore decreasing cGMP production and cGMP concentration. It could inhibit forskolin-induced stellation by decreasing intracellular cGMP concentration which could reduce the inhibition of PDE-3 and therefore increase cAMP degradation.

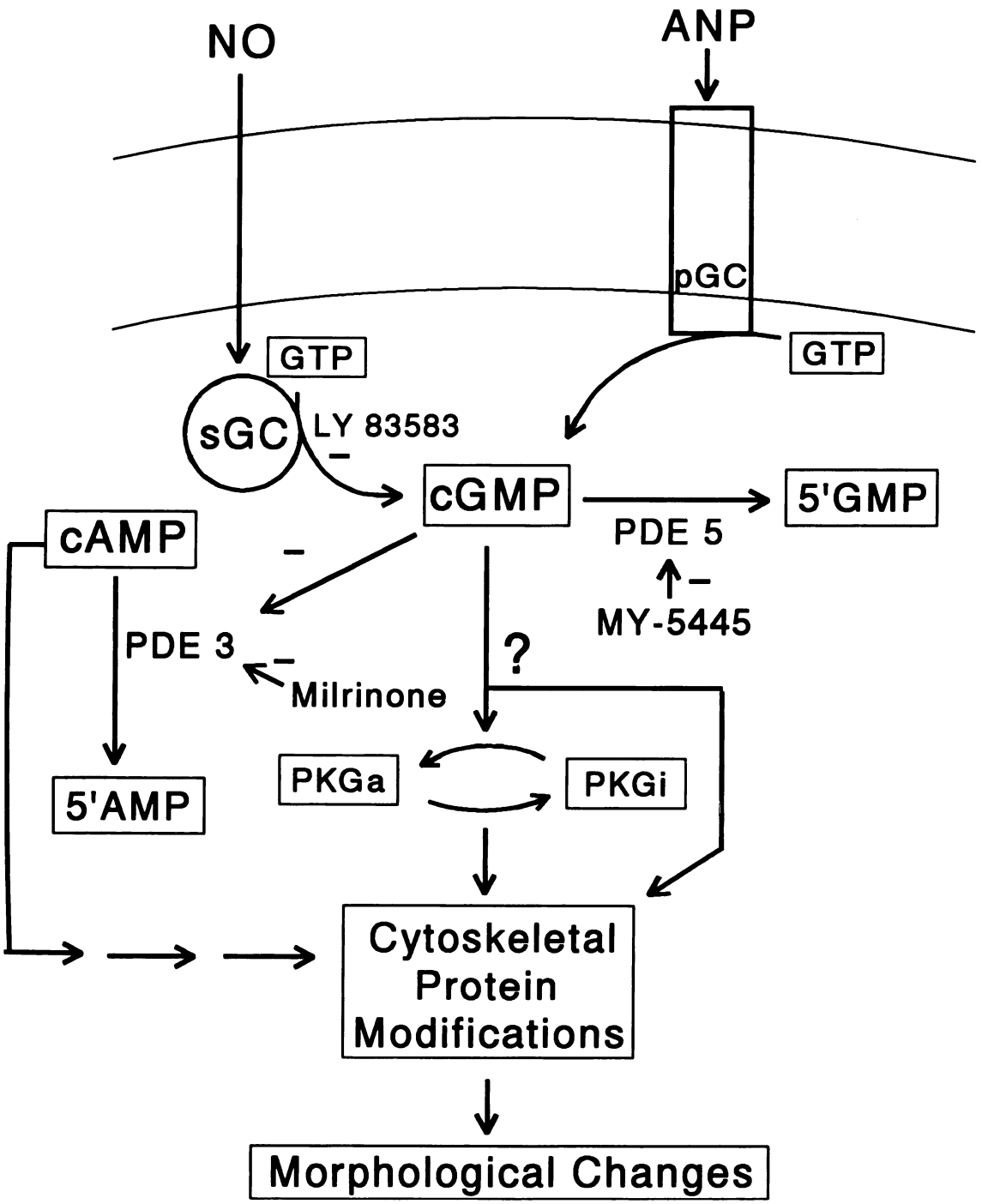

NO
ANP
pGC
GTP
GTP
sGC
LY 83583
cGMP
5'GMP
PDE 5
MY-5445
cAMP
PDE 3
Milrinone
?
PKGa
PKGi
5'AMP
Cytoskeletal Protein Modifications
Morphological Changes

Figure 25

3. Atrial Natriuretic Peptide

To investigate whether atrial natriuretic peptide (ANP) could affect pituicyte morphology *in vitro*, pituicytes were incubated in HBSS containing 1 μM ANP. Pituicyte cultures which were incubated in HBSS containing ANP had a significantly larger fraction of stellate cells compared to cultures incubated in HBSS alone (Figure 24). Results from this study support the hypothesis that ANP can alter the morphology of cultured pituicytes.

C. Discussion

Nitric oxide is proving to be an important regulatory molecule in a variety of cell types in various regions of the body, including the brain. Nitric oxide has been shown to activate soluble guanylate cyclase in the brain which results in increased intracellular cGMP concentration (Katsuki *et al.*, 1977; Miki *et al.*, 1977). Cyclic GMP can activate cGMP-dependent serine-threonine kinase, cGMP-dependent cation channels (Light *et al.*, 1989; Fesenko *et al.*, 1991), and cGMP-dependent phosphodiesterases (Beavo and Reifsnyder, 1990). cGMP-induced activation of cGMP-stimulated phosphodiesterase (PDE 2) can cause a decrease in the intracellular concentration of cAMP (Doerner and Alger, 1988), whereas cGMP-mediated inhibition of cGMP-inhibited phosphodiesterase (PDE 3) has the potential to increase intracellular levels of cAMP (Beavo and Reifsnyder, 1990). For example, one study has demonstrated a synergistic effect between adenylate cyclase and SIN-1 to elevate platelet cAMP concentration (Fisch *et al.*, 1995). In this case, SIN-1 was shown to increase cGMP via activation of soluble guanylate cyclase which inhibited cGMP-inhibited phosphodiesterase (PDE 3) to increase intracellular cAMP concentration. Since the cyclic

nucleotides cAMP and cGMP have the potential to modulate one another, one must consider the possibility that cGMP-induced changes in pituicyte morphology may be mediated by altering the intracellular concentration of cAMP.

As there have been no previous reports in the literature demonstrating that nitric oxide can alter the morphology of primary astrocyte cultures or of astrocytes derived from glial cell lines, the novel finding that nitric oxide donors can affect the morphology of cultured pituicytes is very exciting. The conclusion that the nitric oxide donors sodium nitroprusside and SIN-1 activate soluble guanylate cyclase and therefore increase intracellular cGMP concentration to affect pituicyte morphology is supported by the finding that a significant fraction of pituicytes became stellate when incubated in HBSS containing the membrane permeable analog 8-bromo cGMP. That the soluble guanylate cyclase inhibitors methylene blue and LY-83,583 prevent SNP-induced stellation further supports the hypothesis that SNP mediates stellation by release of NO and activation of soluble guanylate cyclase. The soluble guanylate cyclase inhibitor methylene blue did not prevent 8-bromo cGMP induced stellation. This result was expected since methylene blue inhibits the production of cGMP but should have no effect on the action of cGMP or cGMP analogs. LY-83,583 was also shown to inhibit forskolin-induced stellation, suggesting that may prevent stellation by inhibiting soluble guanylate cyclase which could therefore decrease the concentration of intracellular cGMP and in turn activate a cGMP-inhibited phosphodiesterase. Activation of this phosphodiesterase could then result in an increased rate of cAMP degradation to prevent stellation.

Incubation of cultured pituicytes with the cGMP-specific phosphodiesterase (PDE

5) inhibitor MY-5445 in HBSS also resulted in stellation of a significant fraction of cells compared to cultures incubated in only HBSS. Since PDE 5 hydrolyzes cGMP specifically, inhibition of cGMP-specific PDE 5 is presumed to reduce cGMP degradation and therefore increase the concentration of intracellular cGMP. The results from this study further indicate that increased cGMP levels can induce stellation of cultured pituicytes. In addition, the fact that MY-5445 was able to inhibit PDE 5 activity and induce stellation by itself indicates that there is tonic production of cGMP and tonic activity of PDE 5 in pituicytes. Whether increased levels of cGMP directly induce stellation by activating protein kinase G for example, or indirectly induce stellation by inhibiting cGMP-inhibited PDE (PDE 3 - hydrolyzes cAMP selectively) which would result in increased levels of intracellular cAMP concentration is unknown.

Milrinone, a specific inhibitor of cGMP-inhibited PDE (PDE 3) (Harrison *et al.*, 1986) was also shown to induce stellation of cultured pituicytes when included in the HBSS experimental solution. Inhibition of cGMP-inhibited PDE activity by milrinone is presumed to mediate stellation via an increase in intracellular cAMP concentration. The fact that stellation occurs when this PDE 3 is inhibited by milrinone indicates that PDE 3 is tonically active in non-stellate pituicytes and therefore maintains low levels of cAMP. A high basal level of cGMP-inhibited PDE activity would therefore indicate that cGMP concentration is normally low in non-stellate cultured pituicytes.

If nitric oxide does affect pituicyte morphology *in vivo*, the most likely source of nitric oxide would be nitric oxide synthase from the magnocellular neuroendocrine axon terminals. The most probable isoform of NOS which would be responsible for NO

production in the HNS would be constituitive neuronal NOS, since ultrastructural changes occur within four hours of dehydration (protein synthesis of iNOS would require more on the order of 7-8 hours) and nNOS has been localized to neurons in the CNS. During activation of the HNS, increased intracellular Ca^{+2} levels in the MNC axon terminals associated with increased hormonal activity could therefore provide the elevated Ca^{+2} required for increased constitutive neuronal NOS activity (Bredt and Snyder, 1990). The hypothesis that nitric oxide may mediate ultrastructural changes when the HNS is activated is supported by the finding that the nitric oxide synthase inhibitor L-NAME can alter neurohypophysial ultrastructural relations in dehydrated rats (Beagley and Cobbett, 1996).

It is interesting to note that nitric oxide synthase has been localized in the adrenal medulla (Bredt *et al.*, 1990) and that nitric oxide (Dohi *et al.*, 1983) and the nitric oxide donor sodium nitroprusside (O'Sullivan and Burgoyne, 1990) stimulate catecholamine release in adrenal medullary preparations. It is possible that NOS in the magnocellular neurons releases nitric oxide to influence release of norepinephrine from catecholamine neurons in the neurohypophysis, which could then in turn potentially regulate pituicyte morphology or function. Norepinephrine has been shown to elevate cGMP concentration in different regions of the brain (Ferrendelli *et al.*, 1975; Ohga and Daly, 1977; Vanecek *et al.*, 1985) and stimulates cGMP formation in C6 glioma cells (Bottenstein and De Vellis, 1978). It is possible that NOS functions to stimulate norepinephrine release and that both NOS and norepinephrine induce production of cGMP to maximize cGMP levels when the HNS is activated.

Atrial natriuretic peptide (ANP) is a peptide which can act at a receptor which has

intrinsic guanylate cyclase activity. Incubation of cultured pituicytes in HBSS containing ANP results in a significantly increased fraction of pituicytes which becomes stellate compared to cultures incubated in HBSS alone. As pituicytes have ANP receptors (Luckman and Bicknell, 1991), it is presumed that ANP acts at these receptors to increase particulate guanylate cyclase activity and to subsequently increase intracellular cGMP concentration. Increased intracellular cGMP levels could therefore mediate stellation of cultured pituicytes in a manner analogous to that observed with an increase in cGMP concentration due to treatment of pituictyes with the nitric oxide donors. As there is evidence that ANP is present in the HNS (Jirikowski *et al.*, 1986) and that pituicytes have ANP receptors (Luckman and Bicknell, 1991), the results from this study suggest that there is a potential role for ANP to modulate pituicyte morphology *in vivo*.

Although increased intracellular cGMP concentration can induce stellation of pituicytes in culture, the subsequent steps in the biochemical pathway mediating stellation have not been determined (Figure 25). The cyclic nucleotides cAMP and cGMP have the potential to modulate one another, so it is possible that an increase in only one of these cyclic nucleotides may be ultimately critical for pituicyte stellation. An increase in one of the cyclic nucleotides could enhance intracellular levels of the other, and the effects of these cyclic nucleotides could be either synergistic or additive. As stellation of cultured pituicytes appears to be modulated by alterations in intracellular cAMP and cGMP, it is most probable that alterations in intracellular cyclic nucleotide concentrations are critical for altering pituicyte morphology *in vivo* and therefore are important for regulating hormone entry into the systemic circulation from the neurohypophysis.

IV. Serum Attenuates and Reverses cAMP Mediated Stellation of Cultured Pituicytes

A. Introduction and Experimental Rationale

As discussed previously, cultured protoplasmic astrocytes derived from immature rat brain (Narumi *et al.*, 1978; Pollenz and McCarthy, 1986) or from gliomas (Koschel and Tas, 1993; Oey, 1975; Shain *et al.*, 1987) can become stellate when treated with agents which elevate intracellular cAMP levels. It has been observed that these agents are less effective in inducing stellation when serum is present (Moonen *et al.*, 1975; Kimelberg *et al.*, 1978), and in some cases serum withdrawal alone can cause astrocytes to convert from a flat, epithelial-like form to a stellate morphology (Lim *et al.*, 1973; Moonen *et al.*, 1975, 1976). These studies indicate that serum may play a role in maintaining astroglial cells in a non-stellate morphology.

Serum and lysophosphatidic acid (LPA; monoacylglycerol 3-phosphate), which is a component of serum, have been shown to reverse β-adrenoreceptor induced morphological changes in C6 rat glioma cells within 10-20 minutes (Koschel and Tas, 1993; Nelson and Simon, 1990; Cavanaugh *et al.*, 1990). In studies on fibroblasts, LPA has been shown to activate phospholipase C (PLC) via activation of a pertussis toxin-insensitive G-protein and consequently stimulate calcium mobilization and activation of protein kinase C (Jalink *et al.*, 1990). Studies on fibroblasts have also demonstrated that LPA can inhibit adenylate cyclase activity via activation of a cell surface membrane receptor which is coupled to adenylate cyclase through a pertussis toxin-sensitive inhibitory G-protein (Murayama and Ui, 1987; Van Corven *et al.*, 1989). LPA was shown to decrease cAMP accumulation in intact Rat-1 fibroblasts by 60-70% within 10 min (Van Corven *et al.*, 1989), and this decrease was shown

to be concentration-dependent and completely blocked by pretreatment with the Gi-protein inhibitor pertussis toxin. There appear to be several possible biochemical mechanisms for serum, via LPA, to affect astroglial morphology.

There are several lines of evidence to indicate that serum may regulate pituicyte ultrastructure *in vivo* and *in situ* and affect the morphology of cultured pituicytes. *In vivo*, the neurohypophysis is exposed to serum and serum components, and retraction of pituicytes in response to dehydration, for example, does not occur for several hours. Ultrastructural changes in the isolated intermediate lobe perfused with HBSS containing isoprenaline solution occur within 60 minutes (Luckman and Bicknell, 1990), and morphological changes of cultured pituicytes incubated in a serum-free solution containing a cAMP-elevating agent occur within an hour or so (see Experimental Studies section I). These studies may indicate that the presence of serum *in vivo* can affect the rate at which ultrastructural changes occur. However, it is possible that serum effects the maximal extent of stellation in addition to affecting the rate of stellation. Bicknell *et al.* (1989) have indicated that stellation of cultured pituicytes can occur in response to serum withdrawal alone, which supports the hypothesis that serum functions to maintain cultured pituicytes in a non-stellate form. Whether serum affects pituicyte morphology by modulating factors which control cAMP production and degradation have been investigated in the following studies.

B. Results

1. Serum Attenuates Forskolin and 8-bromo cAMP Induced Stellation of Cultured Pituicytes

A set of experiments was performed to investigate whether serum would attenuate stellation of cultured pituicytes in response to agents which elevate intracellular cAMP concentration. Pituicytes which were incubated for 90 min in HBSS containing 5 μM forskolin (For) had a significantly larger fraction of stellate cells compared to those incubated in HBSS alone (Con) (Figure 26). Addition of 0.5% newborn calf serum to the 5 μM forskolin-containing HBSS solution resulted in a significantly smaller fraction of stellate pituicytes compared to pituicytes incubated in HBSS supplemented with only 5 μM forskolin (For) (Figure 26). Interestingly, pituicytes incubated in HBSS supplemented with 0.5% serum (NCS) had a significantly smaller fraction of stellate cells compared to cultures incubated in HBSS alone (Con) (Figure 26), indicating that there is a basal amount of stellation of cultured pituicytes when serum is removed. These results demonstrate that serum can attenuate forskolin-induced stellation of cultured pituicytes.

Pituicyte cultures incubated in HBSS supplemented with the membrane permeable cAMP analog 8-bromo cAMP (8bcAMP; 150 μM) for 90 min had a significantly larger fraction of stellate cells compared to cultures incubated in HBSS alone (Con) as shown in Figure 27. Inclusion of 0.5% serum in HBSS containing 150 μM 8-bromo cAMP caused a significant decrease in the fraction of cultured pituicytes which was stellate compared to pituicyte cultures which were incubated in HBSS supplemented with only 150 μM 8-bromo cAMP (Figure 27). Results from this study indicate that serum can also attenuate 8-bromo

Figure 26. Serum attenuates forskolin-induced stellation of cultured pituicytes. Pituicyte cultures incubated in HBSS containing forskolin (For; 5 μM) had a significantly larger fraction of stellate cells compared to cultures incubated in HBSS alone (Con). There was a significant decrease in the fraction of pituicytes which was stellate when 0.5% newborn calf serum was included in the forskolin-containing HBSS medium (For+NCS) compared to cultures incubated in HBSS containing only 5% forskolin (For). Treatment of pituicytes with HBSS supplemented with only 0.5% serum (NCS) resulted in a significantly smaller fraction of stellate pituicytes compared to cultures treated with HBSS alone (Con). (✱ - significantly different from HBSS alone (Con); ▲ - significantly different from forskolin alone (For); $p < 0.05$).

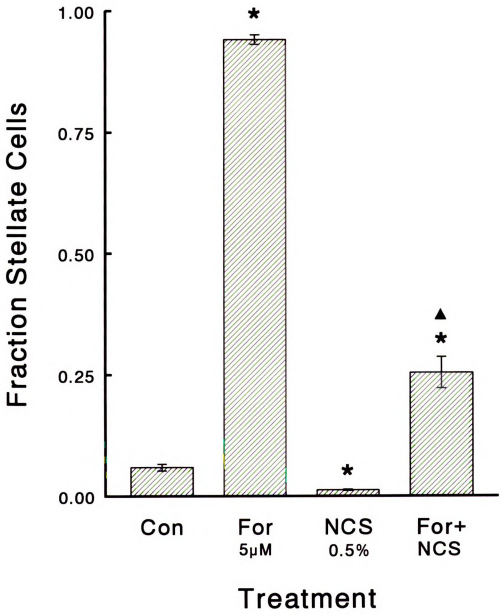

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
▲
*
Con
For
5μM
NCS
0.5%
For+
NCS
Treatment

Figure 26

Figure 27. Serum attenuates 8-bromo cyclic AMP-induced stellation of pituicytes in culture. There was a significant increase in the fraction of pituicytes which became stellate when cultures were incubated in HBSS containing either the membrane permeable cAMP analog 8-bromo cAMP (8bcAMP; 150 μM) or forskolin (For; 5 μM) compared to cultures incubated in HBSS alone (Con). The amount of stellation observed was significantly reduced when 0.5% serum was included in HBSS containing either 150 μM 8-bromo cAMP (8bcAMP+NCS) or 5 μM forskolin (For+NCS) compared to cultures incubated in HBSS containing only 150 μM 8-bromo cAMP (8bcAMP) or 5 μM forskolin (For) respectively. HBSS supplemented with only 0.5% serum (NCS) resulted in a significantly smaller fraction of stellate pituicytes compared to pituicytes incubated in HBSS alone (Con). (✱ - significantly different from HBSS alone (Con); ▲ - significantly different from forskolin (For); ○ - significantly different from 8bcAMP; $p < 0.05$).

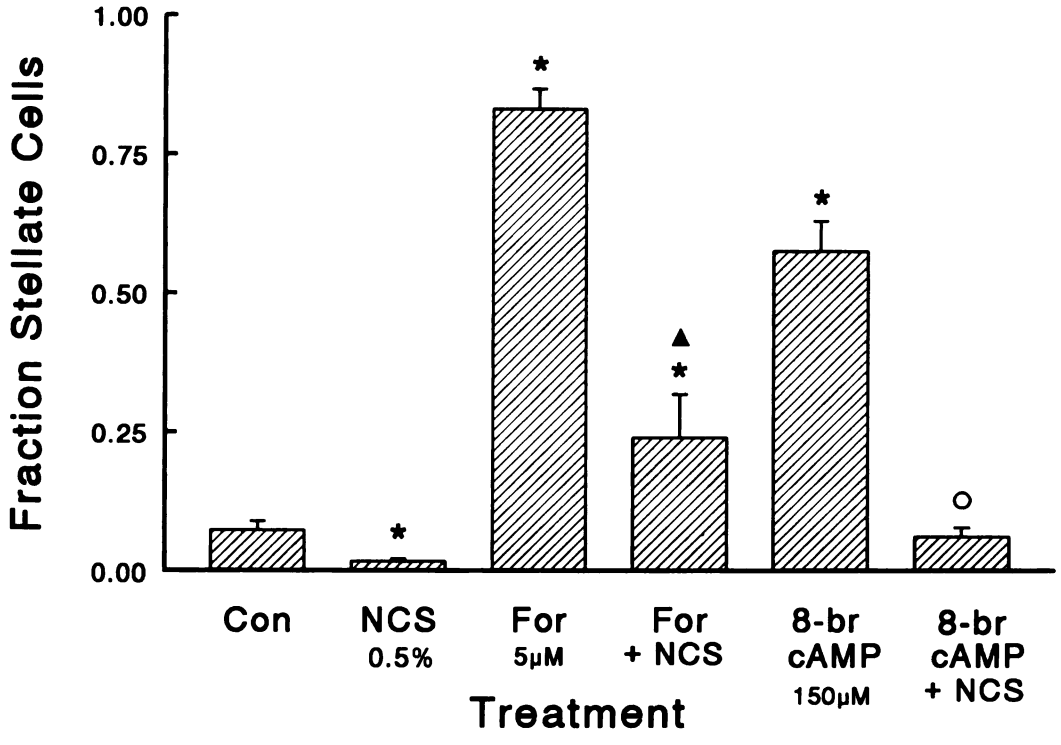

Fraction Stellate Cells
1.00
0.75
0.50
0.25
0.00
Con
NCS
0.5%
For
5μM
For
+ NCS
8-br
cAMP
150μM
8-br
cAMP
+ NCS
Treatment

Figure 27

cAMP induced stellation of pituicytes in culture. Data from these studies demonstrate that serum can attenuate cAMP-mediated stellation of cultured pituicytes.

2. Serum Attenuates IBMX and PTX Induced Stellation of Pituicytes in Culture

As shown previously, the non-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) and pertussis toxin (PTX), an inhibitor of inhibitory G-proteins, can induce stellation of cultured pituicytes (see Experimental Studies section II). Studies were performed to investigate whether serum could attenuate forskolin-induced stellation of cultured pituicytes by increasing phosphodiesterase activity to increase the rate of cyclic nucleotide degradation or by activating an inhibitory G-protein to reduce the activity of adenylate cyclase and therefore decrease cAMP production.

Pituicyte cultures which were to be treated with an experimental solution containing PTX were pretreated with 100 ng/ml PTX (added to the culture medium) 4 hr prior to experimentation. This was done to allow a sufficient period of time for PTX to ADP-ribosylate and therefore inhibit Gi-proteins. As shown in Figure 28, pituicyte cultures incubated in HBSS containing 100 ng/ml PTX for 90 min had a significantly larger fraction of stellate cells compared to pituicyte cultures incubated in HBSS alone (Con). Treatment of cultured pituicytes with HBSS containing both PTX (100 ng/ml) and serum (0.5%) resulted in a significantly smaller fraction of stellate pituicytes compared to pituicytes incubated in HBSS containing only 100 ng/ml PTX (Figure 28). Also, the fraction of pituicytes which was stellate was significantly smaller in cultures incubated in HBSS containing 5 μM forskolin, 0.5% serum, and 100 ng/ml PTX as compared to cultures

Figure 28. Pertussis toxin (PTX) does not alter the effects of serum on pituicyte morphology. Incubation of pituicyte cultures in HBSS containing pertussis toxin, an inhibitor of inhibitory G-proteins (PTX; 100 ng/ml), or in HBSS containing 5 μM forskolin (For) resulted in a significantly larger fraction of stellate cells compared to those incubated in only HBSS (Con). When 0.5% serum was included in HBSS containing 100 ng/ml PTX (NCS+PTX) or 5 μM forkolin (For+NCS) there was a significantly smaller fraction of pituicytes which became stellate compared to cultures incubated in HBSS supplemented with only 100 ng/ml PTX or 5 μM forskolin (For) respectively. There was a significant decrease in the fraction of pituicytes which was stellate when cultures were incubated in HBSS supplemented with 5 μM forskolin, 0.5% serum, and 100 ng/ml PTX (For+NCS+PTX) compared to cultures incubated in HBSS supplemented with only forskolin (5 μM) and 100 ng/ml PTX (For+PTX). (✱ - significantly different from HBSS alone (Con); ▲ - significantly different from forskolin (For); ○ - significantly different from PTX; ■ - significantly different from each other; $p < 0.05$).

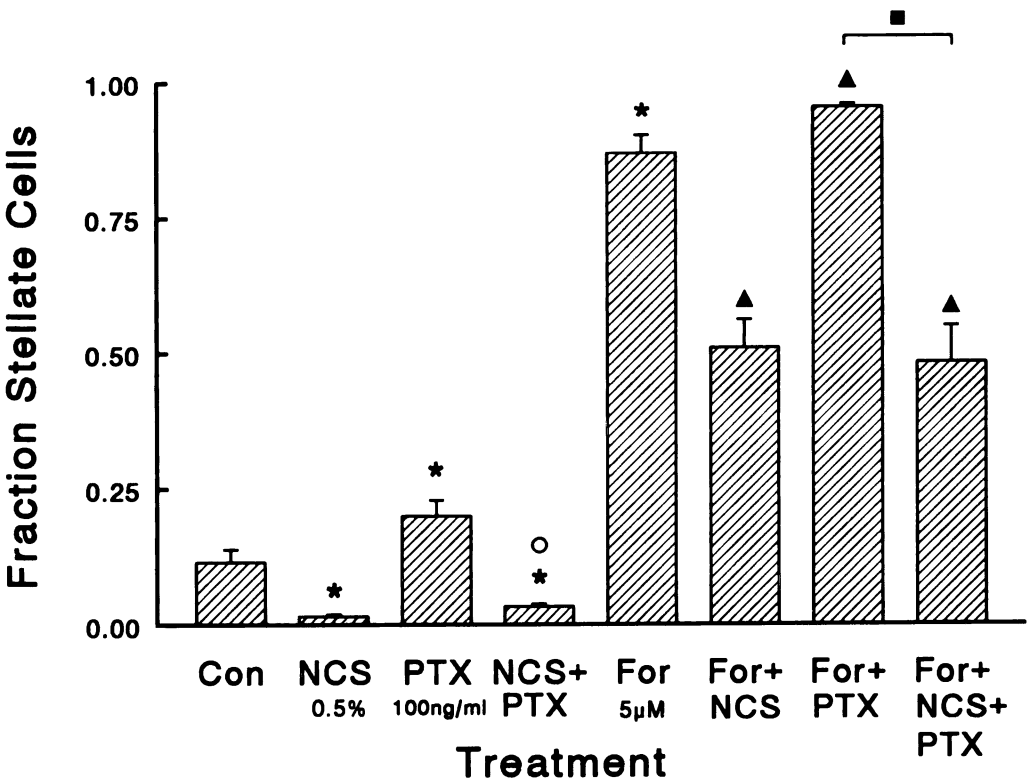

Fraction Stellate Cells
1.00
0.75
0.50
0.25
0.00
Con
NCS 0.5%
PTX 100ng/ml
NCS+ PTX
For 5μM
For+ NCS
For+ PTX
For+ NCS+ PTX
Treatment

Figure 28

incubated in HBSS containing only forskolin (5 μM) and PTX (100 ng/ml) (Figure 28). That serum attenuated PTX-induced stellation and also attenuated forskolin-induced stellation even in the presence of PTX indicates that serum does not act via an inhibitory G-protein to mediate its effects on pituicyte morphology *in vitro*.

The non-specific phosphodiesterase inhibitor IBMX (100 μM) induced a significant fraction of pituicytes to become stellate when added to the HBSS experimental medium compared to cultures incubated in HBSS alone (Con) (Figure 29). Inclusion of 0.5% serum in HBSS containing 100 μM IBMX resulted in a significantly smaller fraction of stellate pituicytes compared to the fraction of pituicytes which became stellate when cultures were incubated in HBSS containing only 100 μM IBMX (Figure 29). The fraction of stellate pituicytes was significantly smaller in cultures incubated in HBSS containing 5 μM forskolin, 0.5% serum, and 100 μM IBMX compared to cultures incubated in HBSS containing only forskolin (5 μM) and IBMX (100 μM) (Figure 29). These results show that IBMX does not alter the effect of serum to attenuate forskolin-induced stellation. Data from these PTX and IBMX studies indicate that serum does not exert its effects on pituicyte morphology via activation of an inhibitory G-protein to decrease adenylate cyclase activity and therefore to decrease cAMP production or by stimulation of phosphodiesterase activity to increase the rate of cyclic nucleotide degradation.

3. Serum Reverses Forskolin-Induced Stellation of Cultured Pituicytes

Once it was established that serum could prevent forskolin-induced stellation of cultured pituicytes, experiments were performed to investigate whether serum could cause

Figure 29. Isobutylmethyl xanthine (IBMX) does not alter the effects of serum on pituicyte morphology. Incubation of pituicyte cultures in HBSS containing the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 100 μM) or in HBSS containing 5 μM forskolin (For) resulted in a significantly larger fraction of stellate cells compared to those incubated in only HBSS (Con). When 0.5% serum was included in HBSS containing 100 μM IBMX (NCS+IBMX) or 5 μM forskolin (For+NCS) there was a significantly smaller fraction of pituicytes which became stellate compared to cultures incubated in HBSS supplemented with only 100 μM IBMX or 5 μM forskolin (For) respectively. There was a significant decrease in the fraction of pituicytes which was stellate when cultures were incubated in HBSS supplemented with 5 μM forskolin, 0.5% serum, and 100 μM IBMX (For+NCS+IBMX) compared to cultures incubated in HBSS supplemented with only 5 μM forskolin and 100 μM IBMX (For+IBMX). (✱ - significantly different to HBSS alone (Con); ▲ - significantly different from forskolin (For); ○ - significantly different from IBMX; ■ - significantly different from each other; $p < 0.05$).

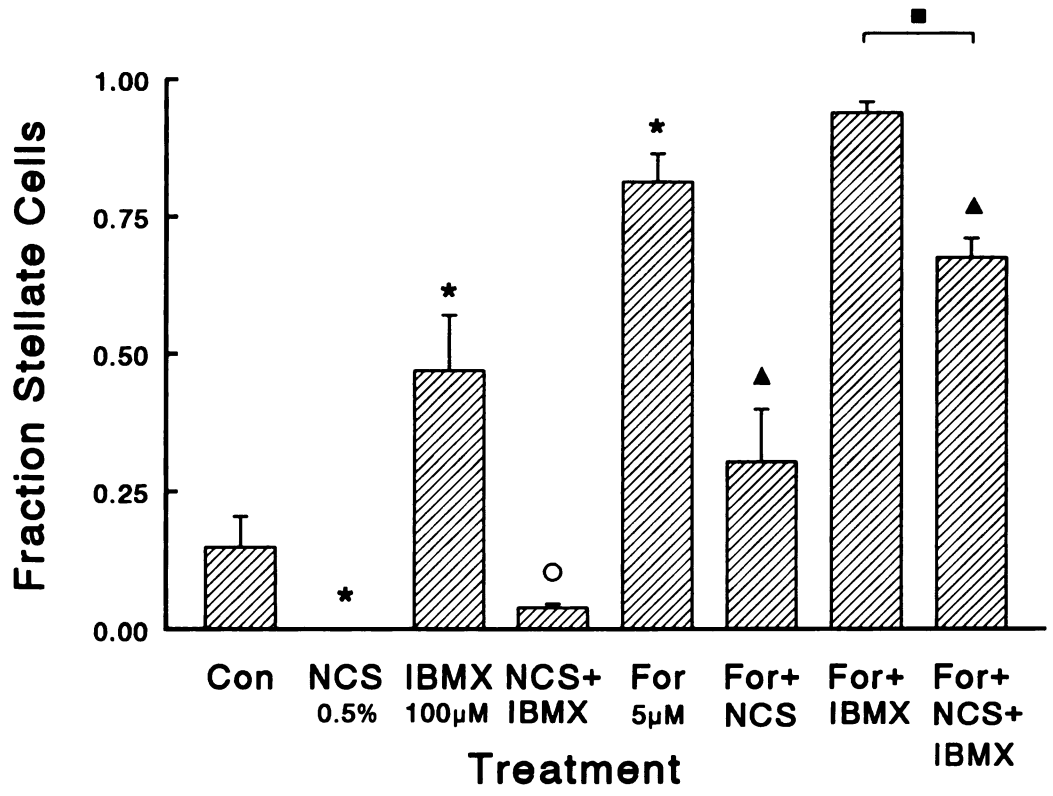

Fraction Stellate Cells
1.00
0.75
0.50
0.25
0.00
Con
NCS 0.5%
IBMX 100µM
NCS+ IBMX
For 5µM
For+ NCS
For+ IBMX
For+ NCS+ IBMX
Treatment

Figure 29

forskolin-induced stellate pituicytes to revert back to a non-stellate morphology. Pituicyte cultures incubated for 90 min in HBSS supplemented with forskolin (F) had a significantly larger fraction of stellate pituicytes compared to cultures incubated in only HBSS for the same period of time (Con) (Figure 30). To determine if serum could induce stellate pituicytes to become non-stellate, cultures were first incubated for 90 min in 5 μM forskolin in HBSS to induce pituicytes to become stellate. Then the cultures were incubated for a consecutive 90 min period in an experimental solution containing either HBSS or HBSS supplemented with 0.5% serum. Cultures which were subsequently incubated with only HBSS remained stellate (Figure 30). However, cultures which were subsequently incubated in HBSS supplemented with 0.5% serum reverted back to the non-stellate morphology and had a significantly smaller fraction of stellate pituicytes compared to cultures subsequently incubated with HBSS alone (Figure 30). The results from this study indicate that serum can cause stellate pituicytes to revert back to a non-stellate morphology.

C. Discussion

Serum and components of serum have been shown to modulate C6 astroglial morphology by reversing cAMP-mediated stellation (Koschel and Tas, 1993; Tas and Koschel, 1990). Some studies have shown that dibutyryl cAMP is less effective in inducing stellation when serum is present (Moonen *et al.*, 1975; Kimelberg *et al.*, 1978). Since serum appears to alter the affects of cAMP-mediated stellation of astrocytes, experiments were performed here to investigate whether serum could both prevent and attenuate forskolin-induced stellation of cultured pituicytes. It was hypothesized that serum would attenuate and

Figure 30. Serum reverses forskolin-induced stellation of pituicytes in culture. Pituicyte cultures incubated in HBSS supplemented with 5 μM forskolin (For) had a significantly larger fraction of stellate cells compared to cultures incubated in only HBSS (Con). Forskolin-induced stellation was not altered when pituicytes were subsequently incubated in HBSS alone (F/C). However, subsequent incubation of forskolin-induced stellate pituicytes with HBSS supplemented with 0.5% newborn calf serum (F/NCS) did result in a significantly smaller fraction of stellate pituicytes compared to cultures subsequently incubated with HBSS alone (F/C). (✱ - significantly different from HBSS alone (Con); ▲ - significantly different from F/C; $p < 0.05$).

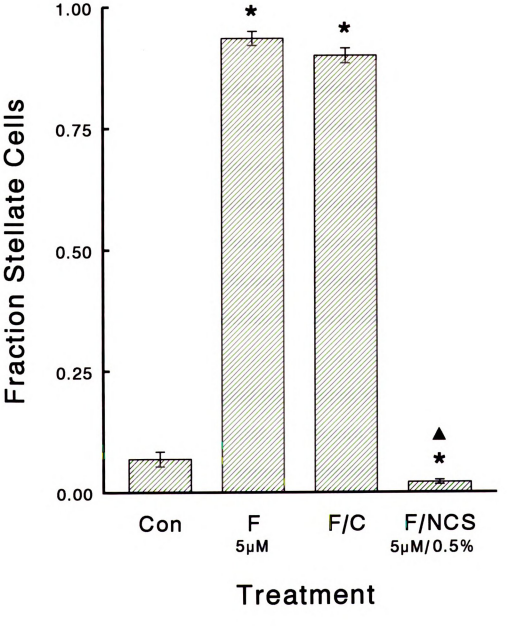

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
▲
*
Con
F
5μM
F/C
F/NCS
5μM/0.5%
Treatment

Figure 30

reverse stellation of cultured pituicytes via 1) activation of an inhibitory G-protein to inhibit adenylate cyclase activity and therefore decrease synthesis of cAMP and levels of intracellular cAMP, or 2) activation of a phosphodiesterase to increase cAMP degradation and therefore decrease intracellular cAMP concentration. However, an alternate mechanism for serum to exert its effects on pituicyte morphology is via modulation of enzymes which directly affect cytoskeletal components involved in controlling pituicyte morphology.

Experimental results presented here demonstrate that serum can both attenuate and reverse cAMP-mediated stellation of cultured pituicytes. That serum attenuated forskolin-induced stellation of cultured pituicytes even when pertussis toxin (PTX), an inhibitor of inhibitory G-proteins, was included in the experimental medium indicates that serum is not acting via an inhibitory G-protein to mediate its effects on pituicyte morphology. There is no endogenous activator of Gi in HBSS, and HBSS containing only PTX induced stellation of cultured pituicytes. These results indicate that there is tonic activity of Gi-proteins in non-stellate pituicytes.

A subsequent study investigated whether serum was increasing phophodiesterase activity to decrease intracellular cAMP concentration to attenuate forskolin-induced stellation. The fact that serum still attenuated forskolin-induuced stellation even in the presence of the non-specific phosphodiesterase inhibitor IBMX indicates that serum does not alter phosphodiesterase activity to affect pituicyte morphology. Treatment of cultured pituicytes with IBMX alone induced stellation, indicating that there is tonic phosphodiesterase activity in non-stellate pituicytes. Results from the PTX and IBMX experiments indicate that serum does not act via activation of an inhibitory G-protein or by

increasing phophodiesterase activity to attenuate stellation. It is reasonable to assume that one biochemical mechanism mediates both attenuation and reversal of cAMP-induced stellation of culture pituicytes by serum and that serum-induced reversal of stellation is not dependent on activation of an inhibitory G-protein or activation of a phosphodiesterase. However, studies here have only looked at whether serum activates a Gi-protein or increases phosphodiesterase activity to attenuate stellation, and therefore further experiments would be necessary to confirm that serum-induced reversal of stellation is also Gi-protein and phosphodiesterase independent.

Pituicytes which had been incubated in HBSS containing forskolin (5 μM) for 90 minutes to induce stellation reverted back to the non-stellate form when subsequently incubated in HBSS supplemented with 0.5% newborn calf serum. Stellate pituicytes did not revert back to the non-stellate form when incubated in HBSS alone. This finding suggests that removal of the stellation-inducing stimulus is not sufficient to cause destellation and that serum must actively initiate destellation to occur in cultured pituicytes.

As mentioned previously, pituicytes become stellate in response to treatment with the membrane permeable cAMP analog 8-bromo cAMP. In this case, G-proteins and adenylate cyclase are circumvented, and pituicyte morphology is altered by a direct increase in intracellular cAMP concentration. Serum was able to attenuate 8-bromo cAMP induced stellation, which suggests that serum acts beyond changes in intracellular cAMP concentration in the biochemical pathway responsible for modulating pituicyte morphology *in vitro*.

Although these results demonstrate that serum modulates the morphology of cultured

Figure 31. Depiction of possible pathways mediating the effects of serum on pituicyte morphology. Serum does not appear to alter cAMP mediated stellation of cultured pituicytes via an inhibitory G-protein ① or by increasing phosphodiesterase activity ②, but rather appears to be acting downstream of changes in intracellular cAMP concentration and therefore modulates enzymes which are more directly involved in regulating cytoskeletal components which alter pituicyte morphology ③. (β2 - beta 2 adrenergic receptor; Gi - inhibitory G-protein; Gs - stimulatory G-protein; AC - adenylate cyclase; PDE - phosphodiesterase; PKA - cAMP-dependent protein kinase; PKG - protein kinase G).

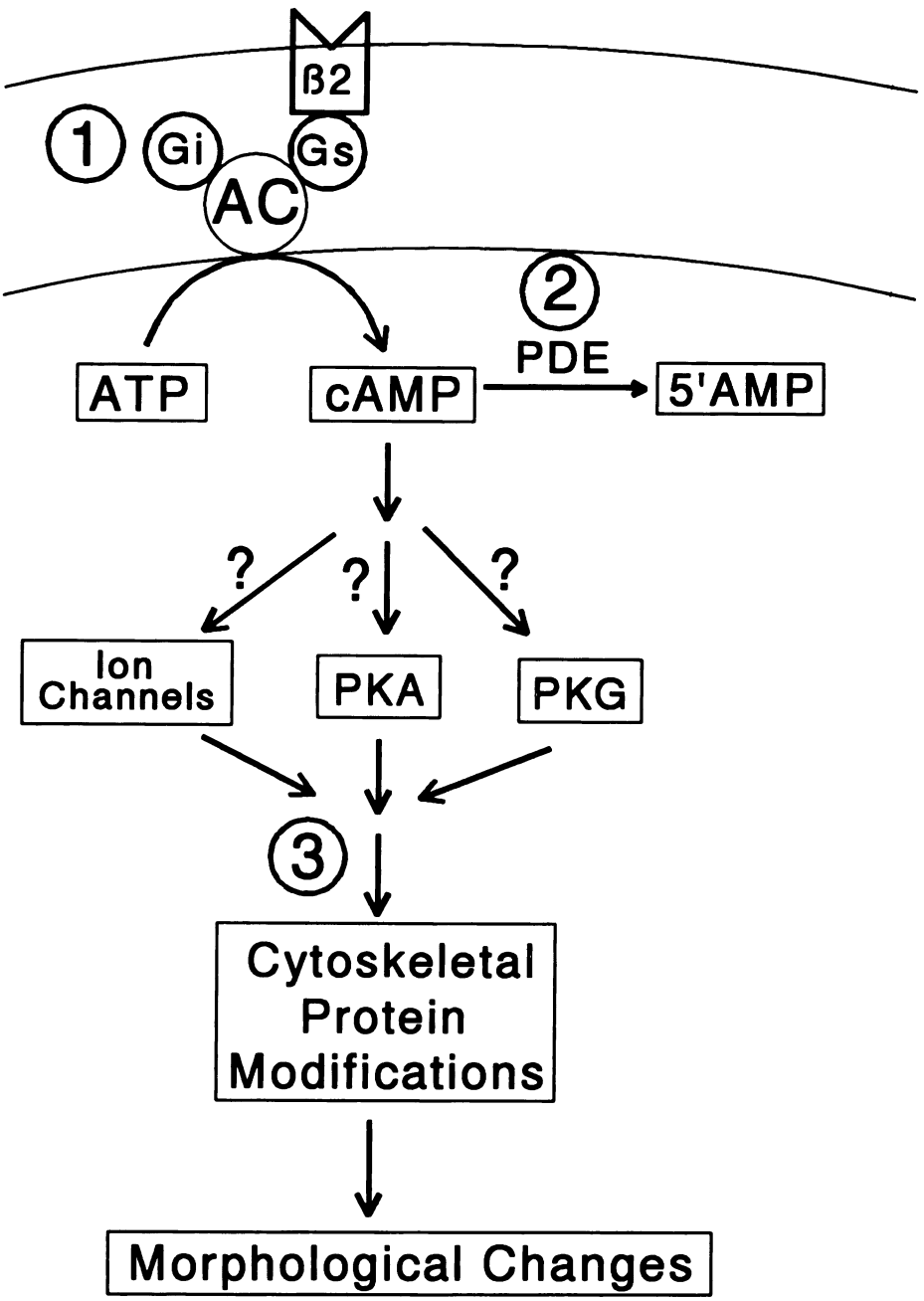

ß2
1
Gi
Gs
AC
2
PDE
ATP
cAMP
5'AMP
?
?
?
Ion Channels
PKA
PKG
3
Cytoskeletal Protein Modifications
Morphological Changes

Figure 31

pituicytes, whether serum plays a role in controlling neurohypophysial neuroglial relations *in vivo* is not known. Since the neurohypophysis is a circumventricular organ and is therefore exposed to serum, there is the potential for serum to affect pituicyte morphology *in vivo*. It appears that ultrastructural changes require a much longer period of time to occur *in vivo* compared to the amount of time it takes for morphological changes to occur *in vitro* supports this notion. Further support for this hypothesis is the finding that perfusion of the brain with hypertonic serum-free medium immediately post mortem induces rapid morphological changes *in situ* (Tweedle *et al.*, 1993). However, since changes in ultrastructure and morphology are most likely both time and concentration dependent, it is possible that serum is altering the extent of stellation in addition to the rate of stellation.

The results from these experimental studies demonstrate that serum can attenuate and reverse cAMP-mediated stellation in cultured pituicytes. These data also indicate that serum does not act via an inhibitory G-protein and does not alter phosphodiesterase activity to affect pituicyte morphology, but rather that serum acts downstream of changes in intracellular cAMP concentration. Serum may regulate enzymes which more directly modulate cytoskeletal components involved in controlling pituicyte morphology (Figure 31). Whether serum affects pituicyte morphology *in vivo* to regulate neurohypophysial hormone secretion will require further investigation.

V. Morphological Changes of Cultured Pituicytes are Independent of Ca^{+2}

A. Introduction and Experimental Rationale

Intracellular calcium acts as a second messenger in response to activation of receptors for a variety of hormones and extracellular growth factors. Alterations in intracellular calcium concentration ($[Ca^{+2}]_i$) regulate cellular activities such as growth and division, metabolism, adhesion, motility, ion transport, and secretion (Bertolino and Llinas, 1992). It has been suggested that changes in $[Ca^{+2}]_i$ play a role in actin polymerization (Stossel, 1989), and several studies have indicated that alterations in the fibroblast cytoskeleton are associated with an increase in intracellular calcium. Several hormones and serum factors have been shown to induce a transient rise in intracellular calcium, and a few studies have indicated that there is a correlation between increased $[Ca^{+2}]_i$ and alterations in cell morphology (Tas and Koschel, 1990; Fawthrop and Evans, 1987).

Calcium mobilization from intracellular stores is involved in the signal cascade triggered by peptide hormones such as bradykinin, endothelin (Quitterer *et al.*, 1995), vasopressin (Mendoza *et al.*, 1986), and bombesin (Zachary *et al.*, 1993). Bombesin and endothelin (1 and 3) have also been shown to induce cytoskeletal reorganization in fibroblasts (Ridley and Hall, 1992) and in primary cultured astrocytes (Koyama *et al.*, 1993; Koyama and Baba, 1994) respectively. Although, recent studies indicate that endothelin-mediated changes in astroglial morphology occur independently of alterations in intracellular calcium, it is possible that peptides which increase intracellular calcium may regulate cell morphology and that alterations in intracellular calcium are involved in inhibiting cAMP-induced stellation of cells in culture.

Although there are no studies demonstrating that vasopressin affects the morphology of cultured pituicytes, vasopressin has been shown to induce mobilization of intracellular calcium (Hatton *et al.*, 1992) and could potentially have an inhibitory effect on stellation. Dynorphin, which has been shown to be colocalized with vasopressin in magnocellular neurons (Watson *et al.*, 1982), can delay and decrease vasopressin-induced mobilization of intracellular calcium, but does not alter basal $[Ca^{+2}]_i$ levels in cultured pituicytes (Boersma *et al.*, 1993). It is possible that dynorphin and vasopressin may be involved in regulating pituicyte structure or function, and future studies may want to address whether vasopressin is involved in maintaining cultured pituicytes a non-stellate morphology.

Serum, lysophosphatidic acid, and thrombin have been shown to increase IP_3 and to induce a transient elevation of intracellular calcium in fibroblasts (van Corven *et al.*, 1989; Jalink *et al.*, 1990) and in C6 glioma cells (Koschel and Tas, 1993). In addition, LPA, serum, and thrombin can reverse β-adrenergic induced stellation of C6 glioma cells. However, destellation was not observed in cells treated with phorbol 12-myristate 13-acetate, which is an activator of protein kinase C, or with Ca^{+2} ionophores (Koschel and Tas, 1993). This study indicates that reversal of stellation of C6 glioma cells in response to treatment with serum and serum components is associated with, but not necessarily dependent on, an increase in intracellular calcium concentration.

Interestingly, some studies have suggested that an increase in $[Ca^{+2}]_i$ is associated with induction of a stellate morphology in cultured astrocytes. Fawthrop and Evans (1987) have reported that calcium ionophores can increase intracellular calcium concentration and cause stellation of cultured astrocytes. It has also been demonstrated that Cd^{+2} and Co^{+2},

which block calcium channels, can inhibit stellation in cultured astrocytes derived from embryonic rat brain (MacVicar, 1987). These findings which indicate that increased $[Ca^{+2}]_i$ can induce stellation of cultured astrocytes seem paradoxical to studies which demonstrate that increased $[Ca^{+2}]_i$ is associated with reversal of stellation.

Fluorescent Ca^{+2} indicator dyes can be utilized to determine changes in intracellular calcium (Thomas and Delaville, 1991). Fura-2 is a particularly useful calcium indicator dye because it enters the cell in an esterified form and is therefore loaded into cells in a non-distruptive manner. One can measure the intensity of light emitted from free fura-2 and from fura-2 bound to calcium in response to excitation at two different wavelengths (350 nm and 380 nm) and then calculate the ratio of bound fura-2 to free fura-2 - the so called "350 nm/380 nm ratio". When there is an increase in intracellular calcium concentration, free fura becomes bound to calcium and thus the ratio of bound fura-2 to free fura-2 increases. Therefore fluorescent Ca^{+2} indicator dyes such as fura-2 provide a convenient and straightforward means for measuring changes in intracellular calcium concentration.

The following studies were performed to investigate if forskolin-induced stellation of cultured pituicytes is associated with an increase in $[Ca^{+2}]_i$ and if the effects of serum to attenuate and reverse forskolin-induced stellation are dependent on an increased level of $[Ca^{+2}]_i$. The first objective was to determine if alterations in $[Ca^{+2}]_i$ occur in response to forskolin and serum, which are substances known to alter pituicyte morphology (see Experimental Studies sections II and IV). Experiments were performed using the fluorescent calcium indicator dye fura-2, and changes in fura-2 fluorescence were monitored to observe alterations in intracellular calcium in response to specified treatment solutions. Changes in

intracellular calcium concentration were also monitored to verify that calcium influx was blocked by HBSS containing cobalt (or nickel) and that intracellular calcium was being depleted by thapsigargin as expected. The second objective was to examine the effect of removal of extracellular Ca^{+2} (by replacing Ca^{+2} with Co^{+2} in HBSS) on forskolin-induced stellation and on attenuation and reversal of forskolin-induced stellation by serum in cultured pituicytes. The third objective was to investigate whether depletion of intracellular Ca^{+2} stores, by inclusion of thapsigargin in calcium-free, cobalt-containing HBSS, would affect forskolin-induced stellation and serum-mediated attenuation and reversal of forskolin-induced stellation. Thapsigargin has been demonstrated to prevent release of intracellular Ca^{+2} by depleting intracellular Ca^{+2} stores via inhibition of a Ca^{+2}-ATPase (Lytton *et al.*, 1991).

B. Results

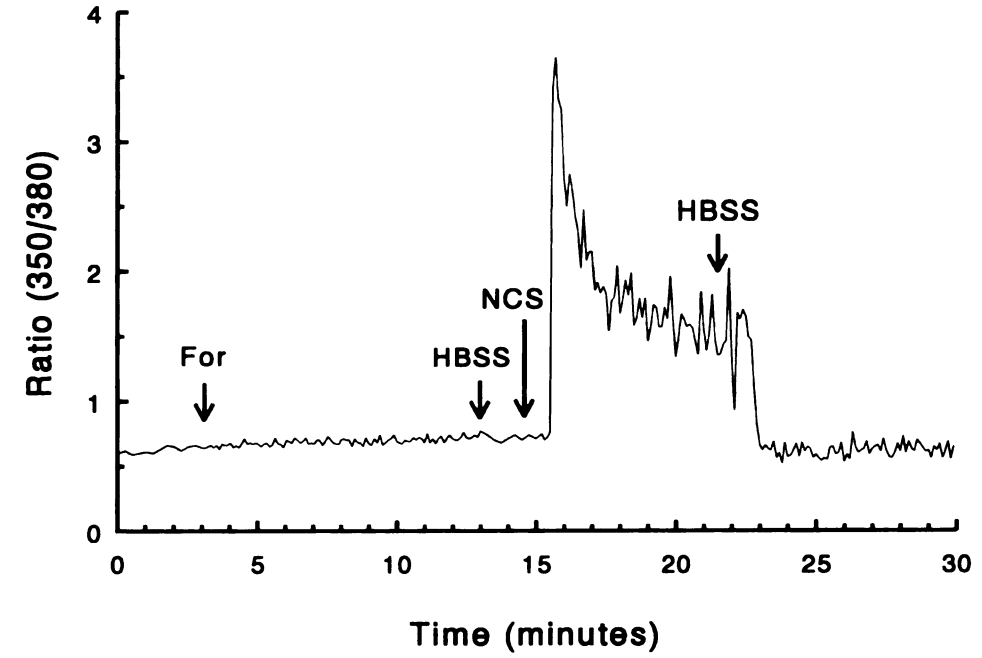
1. Release of Intracellular Calcium is Induced by Serum, but not Forskolin, in Cultured Pituicytes as Indicated by Fura-2

An initial experiment was done to determine if a change in intracellular Ca^{+2} could be induced by forskolin (5 μM) or newborn calf serum (1%), which are both agents that regulate pituicyte morphology *in vitro*. Alterations in intracellular calcium were observed by using the fluorescent calcium indicator dye fura-2. A baseline fluorescence was first recorded for 3 min as the cells were perfused with warm HBSS. The HBSS perfusion buffer was exchanged for an HBSS solution containing 5 μM forskolin and the cells were perfused for 5 min. No change in intracellular calcium was detected in response to perfusion with 5

μM forskolin, indicating that forskolin does not alter the intracellular calcium concentration in cultured pituicytes (Figure 32). Pituicytes were then perfused with HBSS alone for 1.5 min to remove the stimulating agent from the perfusion chamber, and then they were subsequently perfused with HBSS supplemented with 1% newborn calf serum (NCS) for 7 min. The fura-2 350 nm/380 nm fluorescence ratio was shown to increase when pituicytes were perfused with HBSS supplemented with 1% serum, indicating that serum induces an increase in intracellular calcium concentration in pituicytes (Figure 32).

A second set of experiments was performed to determine if pituicytes could respond to treatment with HBSS containing 0.5% serum (demonstrated by an increase in intracellular Ca^{+2} concentration) twice during the same treatment period. Cells were initially perfused with only warm HBSS for 5 min to establish a baseline reading. The HBSS solution was replaced with HBSS supplemented with NCS (0.5%), and pituicyte cultures were perfused for 5 min with 0.5% serum. Alterations in the fura-2 350 nm/380nm fluorescence ratio again indicated that perfusion of pituicytes with serum can induce an elevation in intracellular calcium concentration (Figure 33). Pituicyte cultures were then perfused with HBSS alone for a total of 20 min to determine if the intracellular Ca^{+2} levels would return to baseline. The 350 nm/380 nm ratio decreased, indicating that intracellular calcium levels can return to baseline when serum is removed (Figure 33). The perfusion solution was again exchanged for HBSS supplemented with serum (0.5%), and the cultures were perfused for 5 min to see if a second increase in $[Ca^{+2}]_i$ could be observed. A second increase in intracellular calcium concentration was indeed observed when pituicytes were perfused with HBSS supplemented with 0.5% serum for a second time (Figure 33). The serum-containing HBSS solution was

Figure 32. Forskolin does not cause an increase in intracellular calcium concentration in cultured pituicytes, but serum does. Changes in the intracellular calcium concentration of fura-2-loaded pituicytes was determined by monitoring changes in the intensity of fura-2 fluorescence. The 350 nm/380 nm ratio (top) was calculated using the levels of intensity of fluorescence of fura-2 for each wavelength (bottom). Intracellular calcium concentration was not altered when pituicytes were perfused with 5 μM forskolin (For) in HBSS for 10 min compared to levels observed before forskolin treatment. However, after a brief perfusion with HBSS to rinse out the forskolin-contianing HBSS, an increase in intracellular calcium was observed when pituicytes were perfused with HBSS containing 1% newborn calf serum (NCS). Intracellular calcium levels returned to baseline when serum was removed and pituicytes were perfused with HBSS alone.


Ratio (350/380)
4
3
2
1
0
For
HBSS
NCS
HBSS
0
5
10
15
20
25
30
Time (minutes)

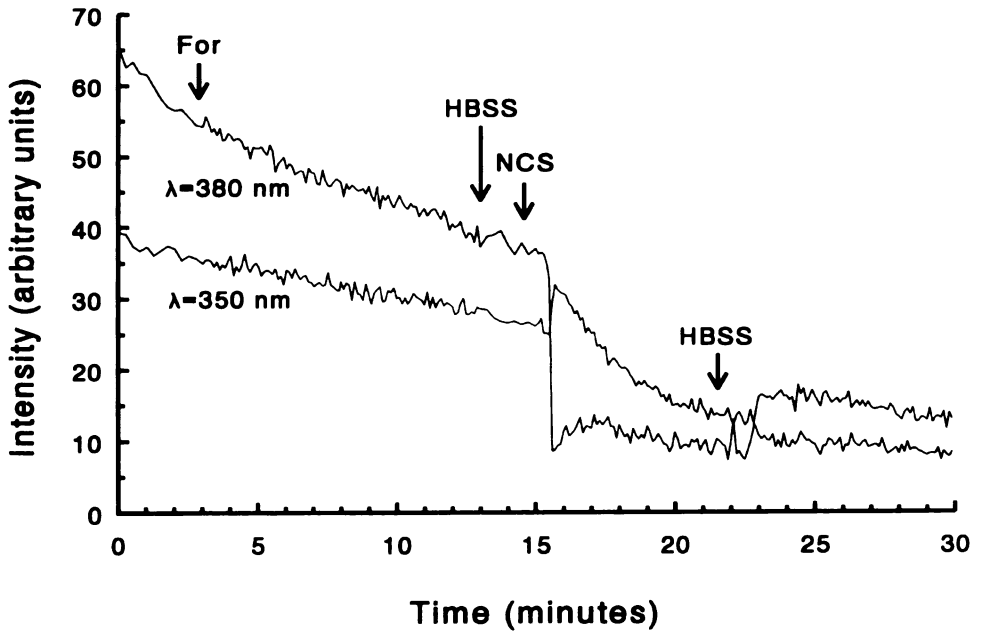
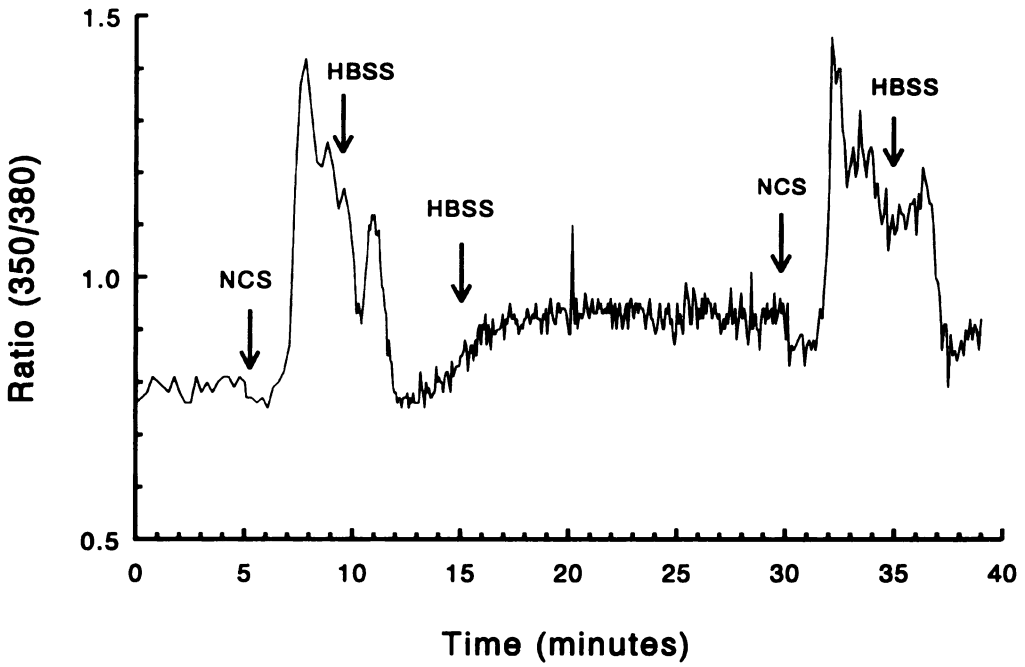

Intensity (arbitrary units)
70
60
50
40
30
20
10
0
For
HBSS
NCS
λ=380 nm
λ=350 nm
HBSS
0
5
10
15
20
25
30
Time (minutes)

Figure 32

Figure 33. Serum induces an increase in intracellular calcium concentration in pituicytes *in vitro*. Pituicytes were perfused with HBSS for 5 min to establish a baseline calcium measurement. When pituicytes were then perfused for 5 min with HBSS containing 0.5% serum (NCS) an increase in intracellular calcium was detected, as the 350 nm/380 nm ratio increased (top). Traces of the light intensities (bottom) indicate that there was an increase in fura-2 bound to calcium and a decrease in free fura. After a subsequent perfusion with HBSS for 15 min, pituicytes were again perfused with HBSS supplemented with 0.5% serum (NCS) and an increase in intracellular calcium concentration was again detected. Pituicytes were then perfused with HBSS for 4 min to demonstrate that the cells could return to baseline intracellular calcium levels. Arrows indicate the time at which the treatment was started. There was a delay of approximately 2 min for the treatment solution to traverse the perfusion line and replace the previous solution in the culture dish.


1.5
1.0
0.5
Ratio (350/380)
NCS
HBSS
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35
40
Time (minutes)

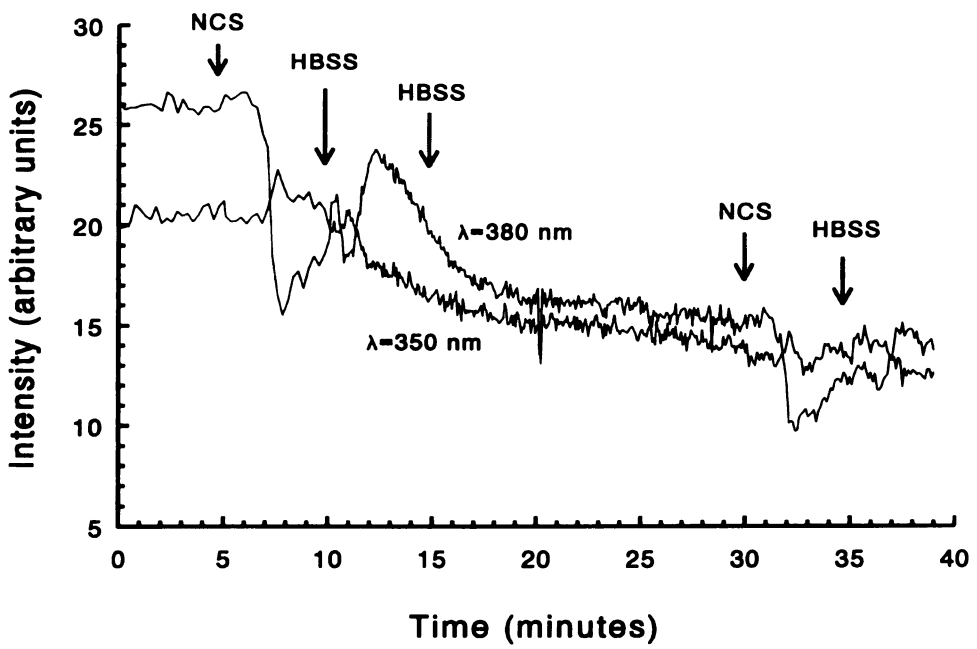

30
25
20
15
10
5
Intensity (arbitrary units)
NCS
HBSS
HBSS
λ=380 nm
λ=350 nm
NCS
HBSS
0
5
10
15
20
25
30
35
40
Time (minutes)

Figure 33

then replaced by HBSS, and pituicyte cultures were perfused for 4 min to observe if intracellular Ca^{+2} levels would again return to baseline readings. The intracellular calcium concentration decreased when pituicytes were perfused with only HBSS, indicating that removal of serum will allow intracellular calcium levels to return to baseline (Figure 33). The results from these studies indicate that serum can induce an elevation in intracellular calcium concentration in cultured pituicytes and that intracellular calcium can be elevated in response to serum more than once in the same pituicyte.

2. Serum-Induced Release of $[Ca^{+2}]_i$ in Cultured Pituicytes Can Be Abolished With Nickel or Thapsigargin

Fura-2 was used to monitor intracellular calcium levels as cultured pituicytes were perfused with HBSS or with a modified calcium-free HBSS solution containing either nickel (2 mM) or nickel and thapsigargin (100 nM). This was done to verify that cobalt and thapsigargin were inhibiting calcium influx and release of intracellular calcium respectively. It should be noted that cobalt interfered with the fura-2 mediated calcium signal, and therefore nickel was used instead. Experiments were performed to determine if the increase in intracellular Ca^{+2} in pituicytes due to treatment with serum could be abolished by either inhibiting Ca^{+2} influx by replacing the $CaCl_2$ in HBSS with $NiCl_2$ or by inhibiting intracellular Ca^{+2} release by depleting internal Ca^{+2} stores with thapsigargin.

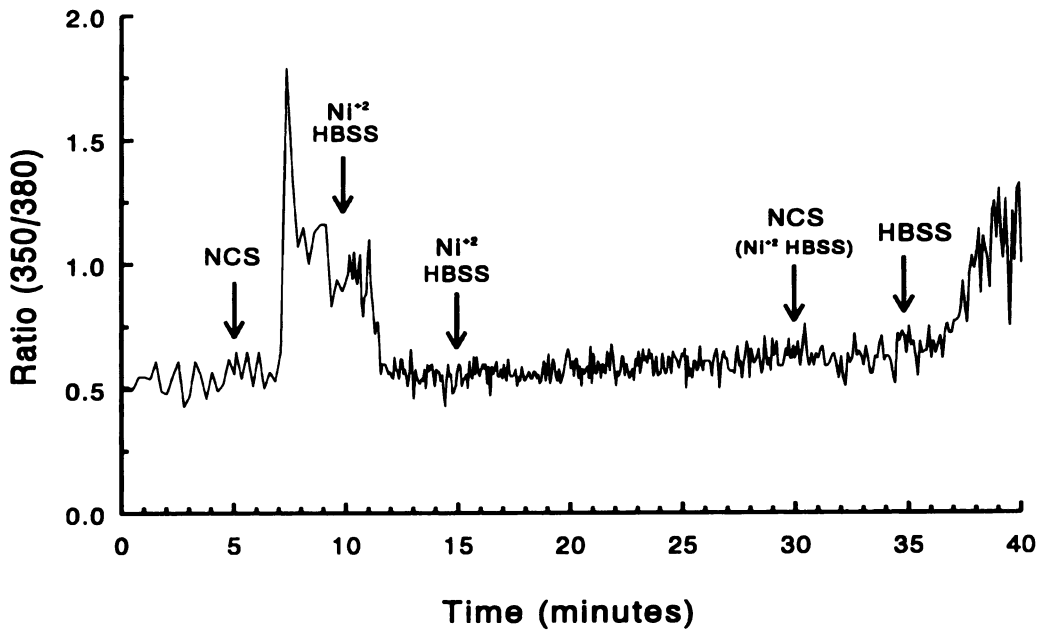
Pituicytes were initially perfused for 5 min with warm HBSS to establish baseline Ca^{+2} levels. This was followed by a 5 min perfusion with HBSS containing NCS (0.5%) to ensure that the object of interest was truely a cell (as opposed to debris) and that it could

respond to treatment with HBSS supplemented with 0.5% serum. Cultures were then perfused with nickel-containing (2 mM; 5 min; calcium-free) HBSS (to remove the NCS-supplemented HBSS) and then with either Ni^{+2}-HBSS (15 min) or with Ni^{+2} - HBSS supplemented with thapsigargin (100 nM; 15 min). Cultures were then perfused with Ni^{+2}-HBSS supplemented with NCS (0.5%) for 5 min to determine whether the pituicyte cultures could still respond to serum by demonstrating an increase in intracellular Ca^{+2}. No elevation in intracellular calcium was observed in pituicytes which had been perfused with nickel-containing HBSS when subsequently perfused with nickel-containing HBSS supplemented with 0.5% serum (Figure 34). As expected, pituicytes which had been perfused with nickel-HBSS containing 100 nM thapsigargin did not have an increased calcium concentration in response to perfusion with modified HBSS supplemented with serum (Figure 35). These studies indicate that depletion of intracellular calcium stores with thapsigargin or perfusion of pituicytes with nickel-containing HBSS can abolish the serum-induced increase in intracellular calcium in cultured pituicytes.

3. The Effects of Forskolin and Serum on Pituicyte Morphology Are Not Altered by Removing Extracellular Ca^{+2} and Blocking Ca^{+2} Channels With Cobalt

Morphology experiments were performed to investigate whether an influx of extracellular calcium was necessary serum to prevent or reverse forskolin-induced stellation of cultured pituicytes. Experimental agents were added to either HBSS (controls) or to a modified HBSS solution where 2 mM $CoCl_2$ was substituted for 2 mM $CaCl_2$. Cultured pituicytes which were incubated for 90 min in HBSS alone were mostly non-stellate, and

Figure 34. Serum does not induce a release of intracellular [Ca^{+2}] in cultured pituicytes in the presence of Ni^{+2}. Fura-2-loaded pituicytes were perfused for 5 min with HBSS and subsequently perfused with HBSS containing serum (0.5%) (NCS) for 5 min. The 350/380 ratio (top) which was calulated from intensities of fura-2 fluorescence (bottom) indicates that 0.5% serum induced a rise in intracellular calcium concentration. Pituicytes were then perfused with calcium-free nickel-containing (2 mM) HBSS for 20 min (5 min and 15 min). When pituicytes were subsequently perfused for 5 min with calcium-free nickel-containing (2 mM) HBSS which was supplemented with serum (0.5%), no change in intracellular calcium concentration was observed.


2.0
1.5
1.0
0.5
0.0
Ratio (350/380)
NCS
Ni+2 HBSS
Ni+2 HBSS
NCS (Ni+2 HBSS)
HBSS
0
5
10
15
20
25
30
35
40
Time (minutes)

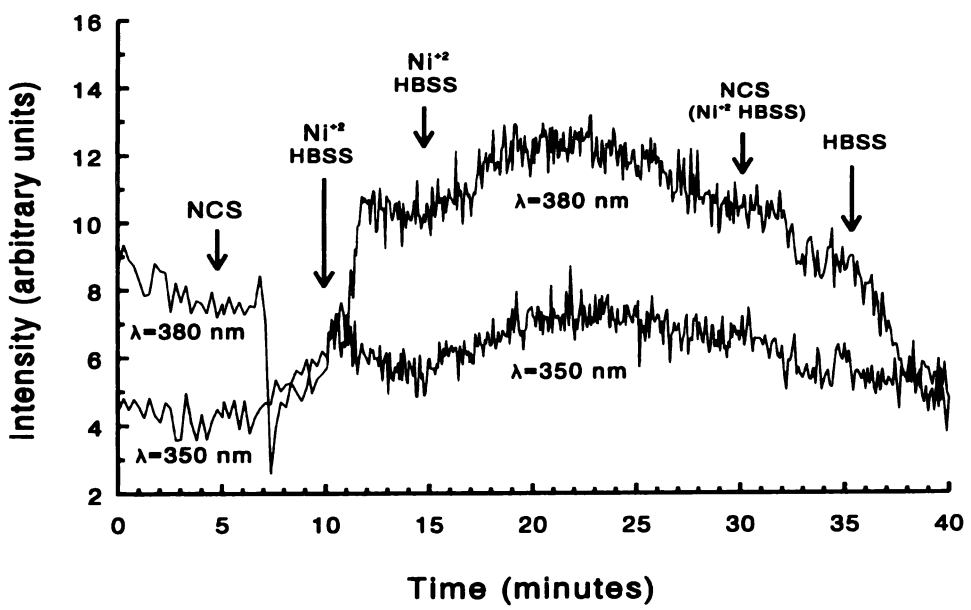
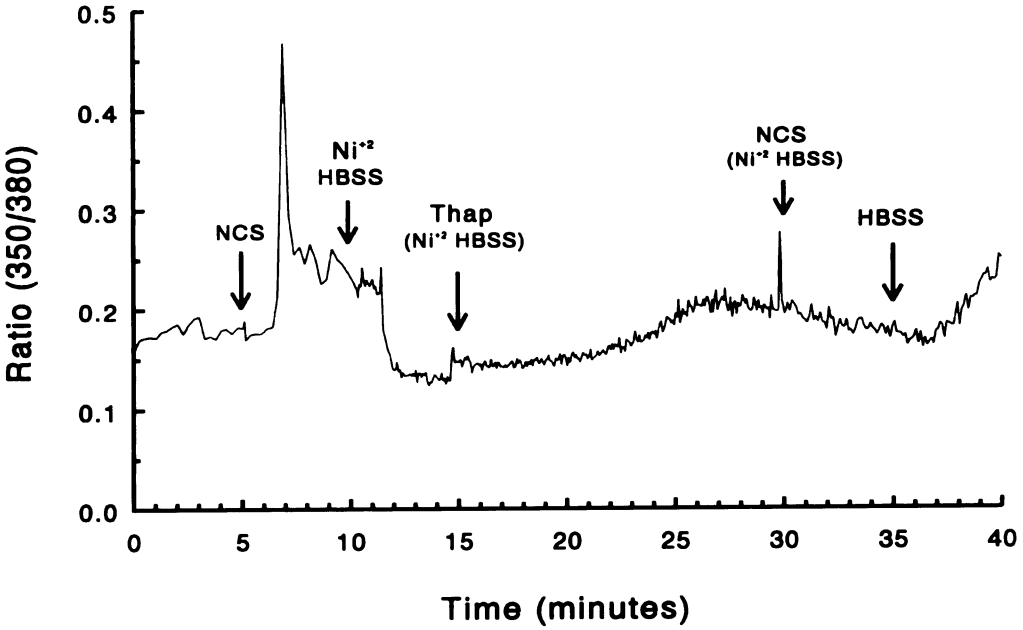

16
14
12
10
8
6
4
2
Intensity (arbitrary units)
NCS
Ni+2 HBSS
Ni+2 HBSS
NCS (Ni+2 HBSS)
HBSS
λ=380 nm
λ=350 nm
λ=380 nm
λ=350 nm
0
5
10
15
20
25
30
35
40
Time (minutes)

Figure 34

Figure 35. Serum does not increase intracellular Ca^{+2} in cultured pituicytes when thapsigargin is present. Pituicytes were perfused for 5 min with HBSS to establish baseline calcium levels. An increase in intracellular calcium was detected when cells were perfused with HBSS supplemented with 0.5% serum (NCS), as the 350/380 ratio for fura-2 fluorescence increased (Top). The ratio was calculated from the intensity of fura-2 fluorescence for 350 nm and 380 nm (Bottom). Pituityes were perfused with calcium-free nickel-containing (2 mM) HBSS for 5 min and subsequently perfused with calcium-free nickel-containing (2 mM) HBSS which was supplemented with 100 nM thapsigargin (Thap) for 15 min. Note that there is a gradual rise in the 350/380 ratio after treatment with thapsigargin, indicating that there is a release of calcium from intracellular stores. Subsequent perfusion of pituicytes with calcium-free nickel-containing (2 mM) HBSS which was supplemented with serum (0.5%) (NCS) did not induce an increase in intracellular calcium concentration.


Ratio (350/380)
0.5
0.4
0.3
0.2
0.1
0.0
NCS
Ni+2 HBSS
Thap (Ni+2 HBSS)
NCS (Ni+2 HBSS)
HBSS
0
5
10
15
20
25
30
35
40
Time (minutes)

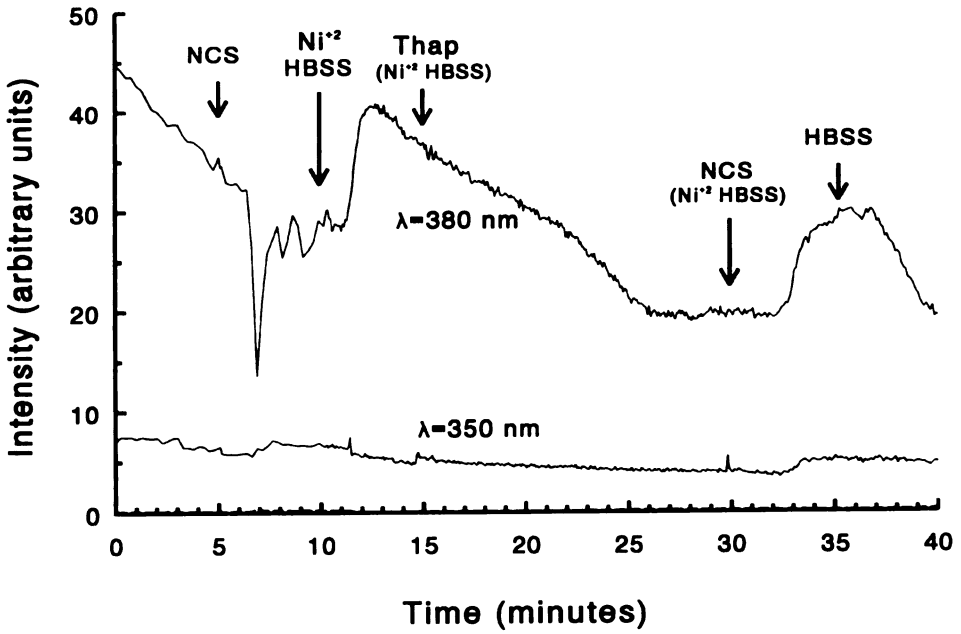

Intensity (arbitrary units)
50
40
30
20
10
0
NCS
Ni+2 HBSS
Thap (Ni+2 HBSS)
NCS (Ni+2 HBSS)
HBSS
λ=380 nm
λ=350 nm
0
5
10
15
20
25
30
35
40
Time (minutes)

Figure 35

those incubated in 5 μM forskolin in HBSS for 90 min were mostly stellate (Figure 36). Replacement of calcium with cobalt (2 mM), which blocks Ca^{+2} channels to prevent calcium influx, in the experimental medium did not affect basal or forskolin-induced (1 μM) stellation (Figure 36). The fraction of cultured pituicytes which was stellate was significantly less in cultures incubated in calcium-containing, cobalt-free HBSS containing 0.5% newborn calf serum compared to cultures incubated in only calcium-containing, cobalt-free HBSS (Figure 36). Replacing calcium with cobalt in the HBSS did not alter the effects of serum (0.5%) alone on pituicyte morphology, and it did not influence the effect of serum to attenuate forskolin-induced stellation (Figure 36).

Experiments were performed to examine the effect of removal of extracellular calcium and inhibition of calcium influx on reversal of stellation by serum. All cultures were first incubated for 90 min in calcium-containing, cobalt-free HBSS containing 5 μM forskolin to induce pituicytes to become stellate. Pituicyte cultures were then incubated for a consecutive 90 min period in calcium-containing, cobalt-free HBSS alone (For/Con) or with 0.5% serum (For/NCS) or in calcium-free, cobalt-containing HBSS (For/Co) alone or with 0.5% serum (For/NSC+Co). Stellate pituicytes remained stellate when incubated in calcium-free, cobalt-containing HBSS (For/Con) (Figure 37). The fraction of pituicytes which was stellate was significantly reduced when stellate cells were incubated in calcium-containing, cobalt-free HBSS supplemented with 0.5% serum, and serum-induced reversal of stellation was not altered when cobalt was substituted for calcium in the HBSS solution (Figure 37).

Figure 36. Co^{+2} does not affect forskolin-induced stellation or attenuation of forskolin-induced stellation by serum in cultured pituicytes. The fraction of pituicytes which was stellate was not significantly different when cultures were incubated in HBSS containing cobalt (Co) as compared to cultures incubated in HBSS alone (Con). Treatment of pituicytes with HBSS supplemented with serum (NCS) had a decreased amount of stellation compared to cultures incubated in only HBSS (Con), and this effect was not altered in the presence of cobalt (NCS+Co). Pituicyte cultures became stellate when incubated in HBSS containing forskolin (For) compared to cultures treated with HBSS alone (Con), and forskolin-induced stellation was not altered in the presence of cobalt (For+Co). Inclusion of serum in HBSS containing forskolin (For+NCS) resulted in a significantly smaller fraction of stellate cells compared to cultures treated with HBSS containing only forskolin (For). Cobalt had no effect on attenuation of forskolin-induced stellation by serum (For+NSC+Co). (✱ - significantly different from HBSS alone (Con); ■ - significantly different from Co; ▲ - significantly different from For; ○ - significantly different from For+Co; NS - not significantly different from each other; $p < 0.05$).

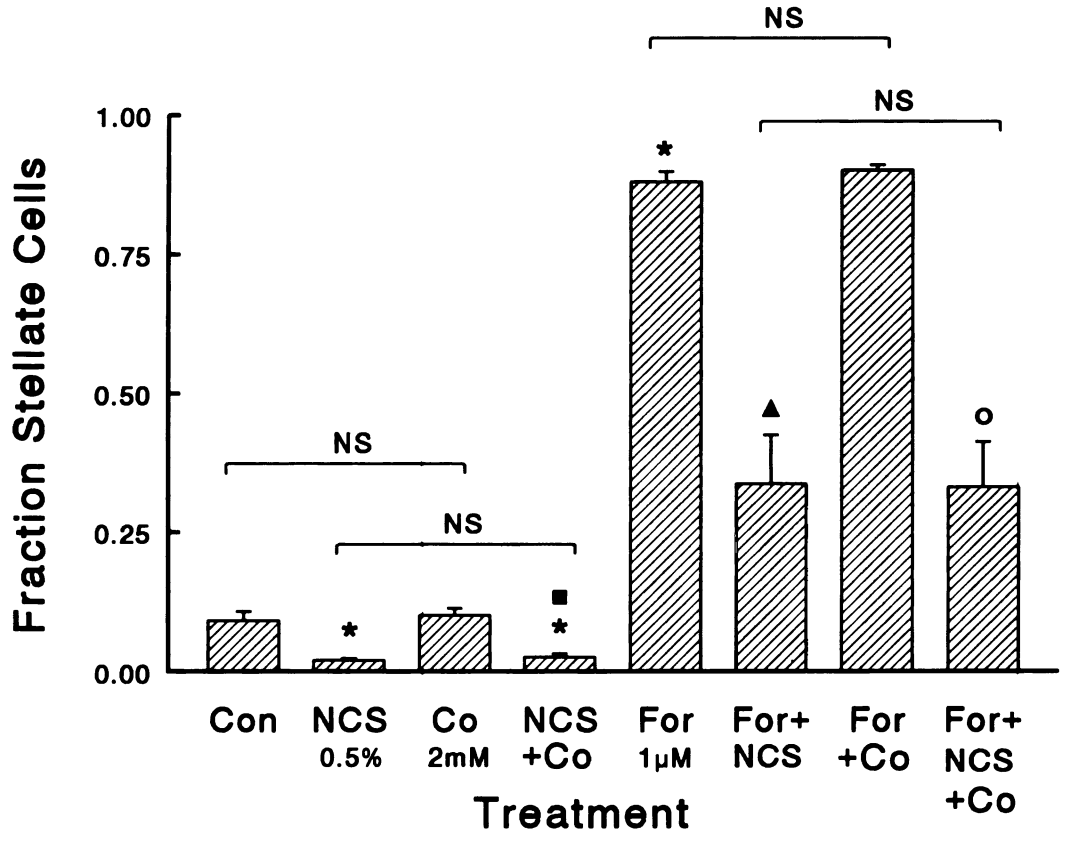

NS
NS
NS
NS
1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
*
■
▲
o
Con
NCS 0.5%
Co 2mM
NCS +Co
For 1μM
For+ NCS
For +Co
For+ NCS +Co
Treatment

Figure 36

Figure 37. Co^{+2} does not reverse forskolin-induced stellation and does not prevent destellation by serum in pituicytes *in vitro*. All pituicyte cultures were first incubated for 90 min in HBSS supplemented with 5 μM forskolin to induce pituicytes to become stellate. Incubation of stellate cells for 90 min in HBSS containing cobalt (For/Co) did not alter the fraction of stellate pituicytes compared to stellate cells treated with only HBSS (For/Con). Destellation of pituicytes occurred when stellate pituicytes were incubated in HBSS containing serum (For/NCS). Cobalt had no effect on reversal of stellation by serum (For/NCS+Co). (✱ - significantly different from For/Con; ■ - significantly different from For/Co; NS - not significant different from each other; $p < 0.05$).

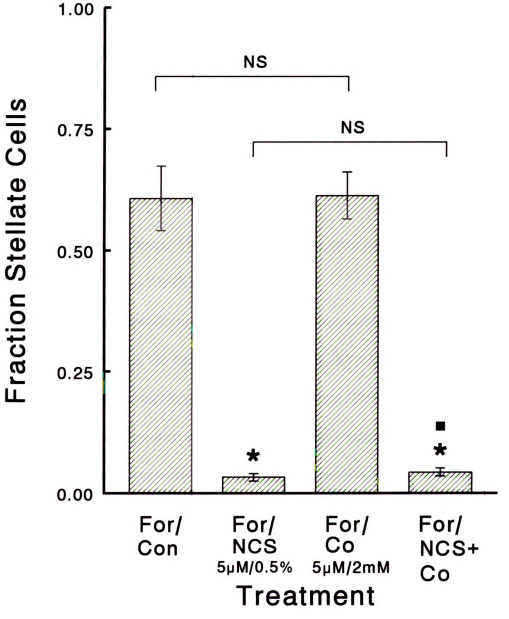

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
NS
NS
*
*
■
For/
Con
For/
NCS
5μM/0.5%
For/
Co
5μM/2mM
For/
NCS+
Co
Treatment

Figure 37

4. Depletion of Intracellular Calcium Stores with Thapsigargin Does Not Alter the Effects of Forskolin and Serum on Pituicyte Morphology

Morphology experiments were performed to investigate whether release of intracellular calcium was necessary for forskolin to induce stellation of cultured pituicytes or for serum to prevent or reverse forskolin-induced stellation. Thapsigargin (100 nM) was added to some experimental solutions to deplete intracellular calcium stores and therefore prevent further release of intracellular calcium, and all experimental solutions were prepared using a modified HBSS solution where 2 mM $CoCl_2$ was substituted for 2 mM $CaCl_2$. Some cultures were pretreated for 30 min in calcium-free, cobalt-containing HBSS supplemented with 100 nM thapsigargin to allow a sufficient period of time for thapsigargin to deplete intracellular calcium stores. The remaining cultures were pretreated for 30 min in calcium-free, cobalt-containing HBSS alone.

Incubation of pituicytes in calcium-free, cobalt-containing HBSS with thapsigargin (100 nM) did not alter the fraction of stellate cells that was observed when cultures were incubated in modified calcium-free, cobalt-containing HBSS alone (Figure 38). Stellation of pituicytes by calcium-free, cobalt-containing HBSS supplemented with forskolin (1 μM) was not altered in the presence of 100 nM thapsigargin (Figure 38), and inclusion of 100 nM thapsigargin in the experimental medium also had no effect on attenuation of forskolin-induced (1 μM) stellation of pituicytes by 0.5% serum (Figure 38). These data indicate that mobilization of intracellular calcium is not required for forskolin-induced stellation of cultured pituicytes or for attenuation of forskolin-induced stellation by serum.

Experiments were performed to examine the effect of depletion of intracellular

Figure 38. Thapsigargin does not alter forskolin-induced stellation or attenuation of forskolin-induced stellation by serum in cultured pituicytes. The fraction of pituicytes which was stellate was not significantly different when cultures were incubated in HBSS containing thapsigargin (Thap) as compared to cultures incubated in HBSS alone (Con). Treatment of pituicytes with HBSS supplemented with serum (NCS) had a decreased amount of stellation compared to cultures incubated in only HBSS (Con), and this effect was not altered in the presence of thapsigargin (NCS+Thap). Pituicyte cultures became stellate when incubated in HBSS containing forskolin (For) compared to cultures treated with HBSS alone (Con), and forskolin-induced stellation was not altered in the presence of thapsigargin (For+Thap). Inclusion of serum in HBSS containing forskolin (For+NCS) resulted in a significantly smaller fraction of stellate cells compared to cultures treated with HBSS containing only forskolin (For). Thapsigargin had no effect on attenuation of forskoin-induced stellation by serum (For+NSC+Thap). (✱ - significantly different from HBSS alone (Con); ■ - significantly different from Thap; ▲ - significantly different from For; ○ - significantly different from For+Thap; NS - not significantly different from each other; $p < 0.05$).

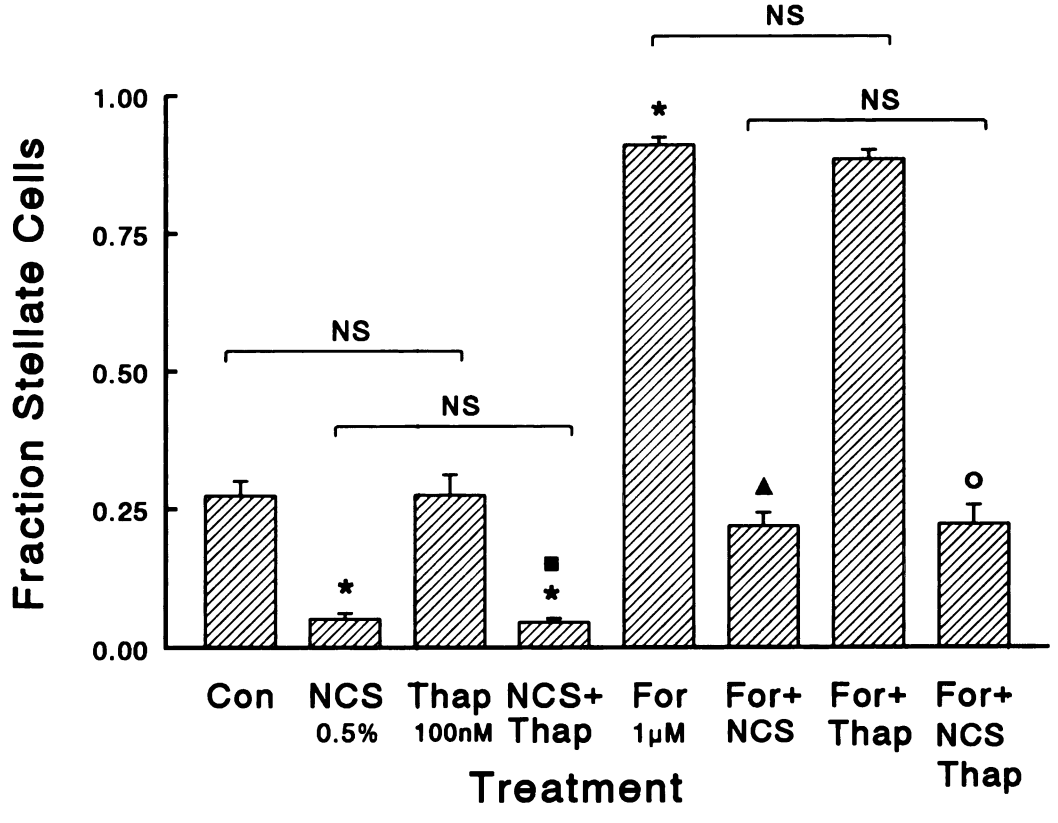

NS
NS
NS
NS
*
*
*
▲
■
o
1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
Con
NCS 0.5%
Thap 100nM
NCS+ Thap
For 1μM
For+ NCS
For+ Thap
For+ NCS Thap
Treatment

Figure 38

Figure 39. Thapsigargin does not reverse forskolin-induced stellation and does not prevent destellation by serum in pituicytes *in vitro*. Pituicytes were initially incubated for 90 min in HBSS containing 5 μM forskolin to induce pituicytes to becomes stellate. Incubation of stellate pituicytes for 90 min in HBSS containing thapsigargin (For/Thap) did not alter the fraction of stellate pituicytes compared to stellate cells treated with only HBSS (For/Con). Destellation of pituicytes occurred when stellate cells were incubated in HBSS containing serum (For/NCS). Thapsigargin had no effect on serum-induced destellation (For/NCS+Thap). (✱ - significantly different from For/Con; ■ - significantly different from For/Thap; NS - not significantly different from each other; $p < 0.05$).

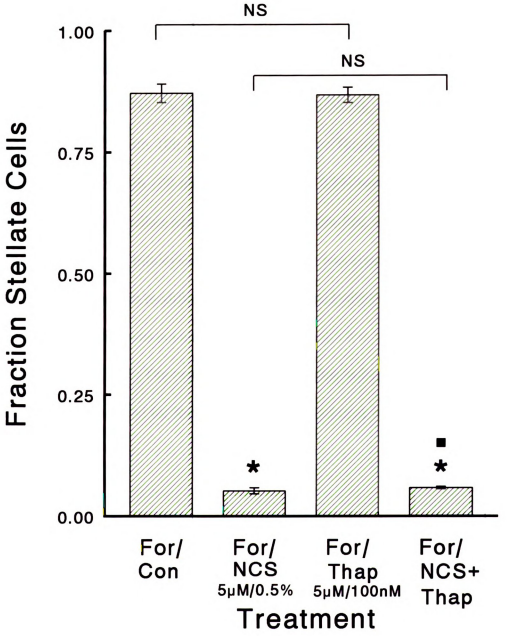

NS
NS
1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
*
*
For/
Con
For/
NCS
5μM/0.5%
For/
Thap
5μM/100nM
For/
NCS+
Thap
Treatment

Figure 39

calcium stores on reversal of stellation by serum. All cultures were first incubated for 90 min in calcium-containing, cobalt-free HBSS containing 5 μM forskolin to induce pituicytes to become stellate. In reversal experiments, all cultures were first incubated for 90 min in calcium-free, cobalt-containing HBSS supplemented with 5 μM forskolin to induce pituicytes to become stellate. Pituicyte cultures were then incubated for a consecutive 90 min period in calcium-free, cobalt-containing HBSS alone (For/Con), or with calcium-free, cobalt-containing HBSS with 0.5% serum (For/NCS), or with calcium-free, cobalt-containing HBSS with 100 nM thapsigargin (For/Thap), or with calcium-free, cobalt-containing HBSS with 100 nM thapsigargin and 0.5% serum (For/NSC+Thap). Stellate pituicytes remained stellate when incubated in calcium-free, cobalt-containing HBSS (Figure 39). The fraction of pituicytes which was stellate was significantly reduced when stellate cells were incubated in modified HBSS supplemented with 0.5% serum, and serum-induced reversal of stellation was not altered when thapsigargin was included in the modified HBSS solution (Figure 39). The results from these studies indicate that release of intracellular calcium is not required for forskolin-induced stellation or for serum to exert its effects on pituicyte morphology.

C. Discussion

Calcium plays an integral role as a second messenger in many cell types and mediates numerous cellular activities in response to stimuli such as neurotransmitters, hormones and growth factors. Several studies have implicated an association between alterations in intracellular calcium concentration and cell morphology. Studies presented here have

investigated whether changes in pituicyte morphology are associated with and /or dependent on alterations in intracellular calcium concentration.

To determine whether experimental treatments altered intracellular calcium concentration, fura-2-loaded pituicytes were treated with forskolin in HBSS or serum in either HBSS or a modified HBSS solution containing nickel or nickel and thapsigargin. Changes in fura-2 fluorescence were monitored as pituicytes were perfused with different experimental media so that the ratio of calcium-bound fura-2 to free fura-2 could be calculated and alterations in intracellular calcium concentration in response to different treatments could be determined. Morphology experiments were performed to assess whether eliminating extracellular calcium or depleting intracellular calcium stores would alter the effect of serum to prevent and reverse forskolin-induced stellation.

The fluorescent calcium indicator dye fura-2 has been utilized to monitor changes in intracellular calcium concentration in pituicytes, and fura-2 experimental data presented here have demonstrated that the intracellular calcium concentration in pituicytes is not altered when they are perfused with HBSS containing 5 μM forskolin. Although studies in the literature have indicated that increased intracellular calcium concentration is associated with stellation of cultured astrocytes (Fawthrop and Evans, 1987; MacVicar, 1987), forskolin-induced stellation of cultured pituicytes is not associated with and is not dependent on changes in intracellular calcium. Data from morphology experiments here demonstrate that blocking calcium influx by replacing calcium with cobalt in the HBSS solution does not alter the ability of forskolin to cause stellation of cultured pituicytes. In addition, depleting intracellular calcium stores with the Ca^{+2}-ATPase inhibitor thapsigargin did not alter the

effects of forskolin to induce stellation of cultured pituicytes. These findings were expected since fura-2 experiments indicated that there was no change in intracellular calcium concentration when pituicytes were perfused with HBSS containing forskolin. Taken together the results from morphology and fura-2 studies, forskolin-induced changes in pituicyte morphology are not associated with changes in intracellular calcium concentration.

Several studies have indicated that serum, LPA, and thrombin induce an increase in intracellular calcium in fibroblasts (van Corven *et al.*, 1989; Jalink *et al.*, 1990) and in C6 glioma cells (Tas and Koschel, 1990; Koschel and Tas, 1993). This increase in intracellular calcium is associated with reversal of stellation in C6 glioma cells, although cytoskeletal changes may not be dependent on alteration in intracellular calcium (Koschel and Tas, 1993). Fura-2 sudies have demonstrated that perfusion of pituicytes with HBSS containing serum results in an increase in intracellular calcium concentration. Monitoring changes in intracellular calcium concentration also verified that intracellular calcium stores are depleted with thapsigargin (a gradual rise in the 350/380 ratio can be observed in response to thapsigargin, indicating a release of internal calcium stores). It was unexpected that removal of extracellular calcium would abolish the serum-induced elevation in intracellular calcium, since it is thought that serum increases intracellular calcium concentration via an IP_3-dependent mechanism and not via an influx of calcium. However, this may be explained by the fact that perfusion in a calcium free solution for a long period of time may have reduced the amount of calcium in the cell and reduced intracellular stores due to the extreme concentration gradient in favor of movement of calcium out of the cell. Regardless of the mechanism of calcium removal from inside the cell, it is apparent from fura-2 experiments

that the effect of serum to increase intracellular calcium concentration was abolished. Results from morphology experiments presented here demonstrate that removing extracellular calcium and depleting intracellular calcium stores have no effect on prevention or reversal of forskolin-induced stellation by serum. This evidence that the serum-induced increase in intracellular calcium was abolished supports data from the morphology experiments indicating that removal of intracellular calcium (whether by removing extracellular calcium or depleting intracellular stores) does not affect the actions of serum on pituicyte morphology.

These studies demonstrate that forskolin-induced stellation is not associated with a change in intracellular calcium and that although the effects of serum on pituicyte morphology are associated with a change in intracellular calcium levels, they are not dependent on alterations in intracellular calcium. The data from these studies support the findings by other investigators who have indicated that changes in astrocyte morphology occur independently of alterations in intracellular Ca^{+2} concentration (Shain *et al.*, 1992; Koschel and Tas, 1993). That several studies appear to have conflicting results may reflect a difference in experimental conditions or an intrinsic difference between cell types derived from different sources, such as cells obtained from different regions of the brain.

It may be hypothesized that serum or a component of serum binds to a receptor which is coupled to phospholipase C (PLC) which stimulates the hydrolysis of phosphatidylinositol 4, 5 bisphosphate (PIP_2) to inositol trisphosphate $(IP)_3$ and diacylglycerol (DAG). Diacylglycerol could then activate protein kinase C to potentially modulate cell morphology, while an IP_3 mediated increase in intracellular calcium is just an associated phenomenon that

is not involved in mediating morphological changes. There is now evidence in the literature indicating that the serum component LPA acts via a receptor which is coupled to stimulation of phospholipase C, inhibition of adenylate cyclase, and to activation of the small GTP-binding proteins Ras and Rho (see Moolenaar, 1995). Recent fibroblast studies have indicated that the serum component LPA binds to a receptor and activates Rho to exert effects on components of the actin cytoskeleton (Ridley and Hall, 1992, 1994). However, there have been no studies demonstrating that LPA affects cell morphology via this mechanism.

VI. A Role for Tyrosyl Phophorylation in Astroglial Morphology

A. Introduction and Experimental Rationale

As previously discussed, studies have demonstrated that serum can reverse cAMP mediated morphological changes in C6 glioma cells (Tas and Koschel, 1990). In addition, treatment of cultured pituicytes with serum can attenuate forskolin-induced stellation and cause stellate pituicytes to revert back to a non-stellate form (see Experimental Studies section IV). Lysophosphatidic acid is one component of serum that has been shown to mediate the morphological effects of serum in C6 glioma cells (Koschel and Tas, 1993). Several studies in fibroblasts have focused on serum- or LPA- induced cytoskeletal changes, although none have investigated the effects of serum or LPA on cell morphology.

Fibroblasts and many other types of cultured cells develop specialized adhesions, known as focal adhesions, to the underlying substratum (Burridge *et al*, 1988). Focal adhesions are rich in the cytoskeletal protein vinculin, and a diversity of proteins, including integrins, cytoskeletal proteins, proteases, protein kinases, protein phosphatases, and other signalling molecules colocalize with vinculin in focal adhesions (Burridge *et al.*, 1988). At their cytoplasmic face, focal adhesions anchor bundles of actin. These bundles of actin are referred to as "stress fibers" and are one of the major cytoskeletal structures in fibroblasts and a variety of other cells (Burridge *et al.*, 1988).

Serum and lysophosphatidic acid have been demonstrated to stimulate actin stress fiber and focal adhesion formation in serum-starved Swiss 3T3 cells (Ridley and Hall, 1992). This has been shown to occur with recruitment of cytoskeletal proteins to newly formed focal adhesions and with tyrosyl-phosphorylation of cytoskeletal proteins, including focal

adhesion kinase (p125FAK) and paxillin (Kumagai *et al.*, 1993; Chrzanowska-Woodnicka and Burridge, 1994; Seufferlein and Rozengurt, 1994; Barry and Critchley, 1994). Genistein, a selective inhibitor of protein tyrosine kinases (Akiyama *et al.*, 1987; Linassier *et al.*, 1989), has been shown to block serum-induced tyrosyl-phosphorylation of focal adhesion kinase and assembly of focal adhesions and stress fibers in quiescent Swiss 3T3 fibroblasts (Chrzanowska-Woodnicka, 1994; Ridley and Hall, 1994), further indicating that tyrosyl-phosphorylation of cytoskeletal proteins is involved in regulation of the actin cytoskeleton.

Although evidence from fibroblast studies suggests that tyrosyl-phosphorylation of cytoskeletal proteins may be important in controlling cell morphology, the relationship between tyrosyl-phosphorylation of cytoskeletal proteins and cell morphology of fibroblasts has never been examined. Since morphological plasticity has been observed in cells derived from gliomas and in primary cultured astrocytes, it was of interest to determine if cellular morphology of astroglial cells could be correlated with the tyrosyl-phosphorylation status of cytoskeletal proteins.

Western Blot analysis was required for examination of tyrosyl-phosphorylation of cytoskeletal proteins in astroglial cells, and therefore a large amount of protein was needed for this investigation. As cultured pituicytes were never confluent on a coverslip and provided only a small amount of protein, pituicytes were used only for morphology experiments and were not used for Western Blot analysis experiments. Instead an astroglial cell line was chosen to perform Western Blot analysis studies and morphology studies. RG-2 cells are non-neoplastic astrocytes derived from immature rat brain in secondary culture (Ko

et al., 1980). Previous studies have determined that RG-2 cells exhibit morphological plasticity which is similar to that observed in cultured pituicytes. RG-2 cells have therefore been used in the present study to examine the association between tyrosyl-phosphorylation and cell morphology.

In the following studies the effects of forskolin, genistein, and serum on pituicyte and RG-2 cell morphology and on tyrosyl-phosphorylation of presumed cytoskeletal proteins in RG-2 cells have been investigated. Western blot analysis was used in conjunction with evaluation of astroglial morphology to determine if there is a correlation between cell morphology and tyrosyl-phosphorylation of presumed cytoskeletal proteins. Morphology experiments were first performed in pituicytes to investigate whether tyrosine kinase inhibition would alter the effects of serum on prevention and reversal of forskolin-induced stellation. Similar morphology experiments were then performed in RG-2 cells to establish whether tyrosine kinase inhibition had similar effects to that observed in pituicyte experiments. In addition, Western Blot analysis was performed using RG-2 cells so that it could be determined if there was an association between cell morphology and the tyrosyl-phosphorylation status of presumed cytoskeletal proteins.

B. Results

1. Genistein Attenuates the Effects of Serum to Prevent and Reverse Forskolin-Induced Stellation of Pituicytes *In Vitro*

Experiments were performed to investigate the effects of genistein, and therefore tyrosine kinase inhibition, on pituicyte morphology *in vitro*. Pituicytes which were

incubated for 90 min in HBSS were mostly non-stellate, and incubation of cultured pituicytes in forskolin-containing (2.5 μM) HBSS resulted in a significantly larger fraction of stellate pituicytes compared to cells incubated in only HBSS (Figure 40). The amount of stellation induced by forskolin was greatly reduced when 0.5% serum (NCS) was present in the medium, as shown previously (Figure 40). Although genistein (50 μM) alone did not induce stellation of cultured pituicytes, inclusion of 50 μM genistein in HBSS containing 2.5 μM forskolin and 0.5% serum resulted in a significantly larger fraction of pituicytes which became stellate compared to cultures treated with HBSS containing only 2.5 μM forskolin and 0.5% serum (Figure 40).

The fraction of stellate pituicytes remained high when pituicytes were incubated in forskolin-containing 5 μM HBSS for 90 min and subsequently incubated for 90 min with HBSS alone (Figure 41). Destellation (reversal of morphology form the stellate to non-stellate form) occurred when stellate pituicytes (previously incubated in HBSS containing 5 μM forskolin) were subsequently incubated in HBSS supplemented with 0.5% newborn calf serum (Figure 41). Although destellation still occured when genistein was present, inclusion of genistein (50 μM) in the HBSS solution supplemented with 0.5% serum for the second 90 min incubation resulted in a significantly reduced amount of destellation as compared to cultures which were subsequently incubated with HBSS containing only 0.5% serum (Figure 41). The data from these experiments indicate that genistein can significantly attenuate both prevention of forskolin-induced stellation by serum and serum-induced destellation of stellate pituicytes in culture. These results suggest that serum acts at least partially through a genistein-sensitive tyrosine kinase to mediate its effects on pituicyte

Figure 40. Genistein reduces the effect of serum to attenuate forskolin-induced stellation in cultured pituicytes. Pituicyte cultures incubated with HBSS containing forskolin (For; 2.5 μM) had a larger fraction of stellate cells compared to cultures incubated with only HBSS (Con). The fraction of pituictyes which was stellate was reduced when serum was included in the forskolin-containing HBSS solution (For+NCS). The amount of stellation was significantly greater when pituicytes were incubated in HBSS containing forskolin, serum, and genistein (For+NCS+Gen) compared to cultures incubated in HBSS containing only forskolin and serum (For+NCS). Genistein alone in HBSS (Gen) did not induce stellation of cultured pituicytes. (✱ - significantly different from HBSS alone (Con); ▲ - significantly different from For; ■ - For+NCS and For+NCS+Gen are significantly different from each other; $p < 0.05$).

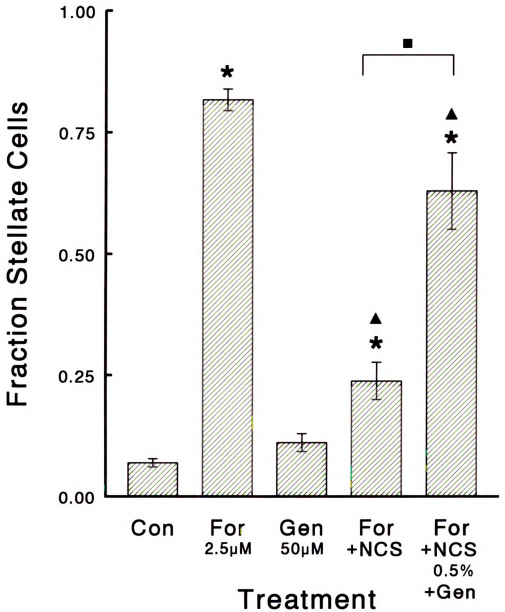

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
Con
For
2.5μM
Gen
50μM
For
+NCS
For
+NCS
0.5%
+Gen
Treatment

Figure 40

Figure 41. Genistein attenuates the effect of serum to reverse forskolin-induced stellation of pituicytes in culture. Stellate pituicytes (pretreated for 90 min with HBSS containing forskolin; 5 μM) remained stellate when subsequently incubated with only HBSS for 90 min (F/C). Stellate pituicytes reverted to a non-stellate form when subsequently incubated in HBSS containing serum (F/N). When genistein was included in the experimental solution for the second treatment (F/N+G), there was a significantly larger fraction of stellate pituicytes compared to cultures subsequently incubated in HBSS with only serum (F/N). (✱ - significantly different from F/C; ▲ - F/N and F/N+G are significantly different from each other; $p < 0.05$).

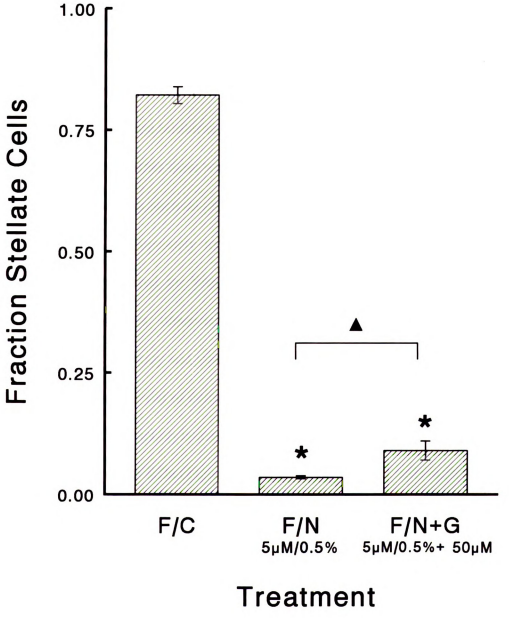

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
▲
*
*
F/C
F/N
5μM/0.5%
F/N+G
5μM/0.5%+ 50μM
Treatment

Figure 41

morphology.

2. Altered RG-2 Cell Morphology Is Associated With Changes in Protein Tyrosyl-Phosphorylation

RG-2 cells were used in the following experiments as an astroglial model to investigate if changes in cell morphology can be correlated with alterations in protein tyrosyl-phosphorylation. An initial set of RG-2 experiments was performed to determine the effects of tyrosine kinase inhibition on prevention of forskolin-induced stellation by serum and to determine whether there were associated alterations in the tyrosyl-phosphorylation status of presumed cytoskeletal proteins. RG-2 cell cultures were incubated for 80 min in HBSS or in an appropriately modified HBSS solution and then either evaluated for morphology or prepared for Western Blot analysis. Tyrosyl-phosphorylation of RG-2 cellular proteins was observed in proteins which were immunoreactive to the phosphotyrosine antibody and visualized using enhanced chemiluminescence (Figure 42). RG-2 cultures directly out of culture medium or incubated in HBSS alone had high levels of protein tyrosyl-phosphorylation of two particular proteins of apparent molecular weight of 60-68 KDa and 125 KDa (Figure 42). Incubation of RG-2 cells in forskolin-containing HBSS (5 μM) resulted in a decreased amount of tyrosyl-phosphorylation of the 60-68 KDa and 125 KDa proteins compared to cells which had been incubated in only HBSS or taken directly out of culture medium (Figure 42). HBSS supplemented with genistein (50 μM) also caused a reduction in the amount of tyrosyl-phosphorylation of the proteins of interest (Figure 42). The forskolin- (For; 5 μM) and genistein- (Gen; 50 μM) induced reduction of

Figure 42. Tyrosyl-phosphorylation of RG-2 cell proteins is demonstrated by immunocytochemical visualization (stellation). Tyrosyl-phosphorylated proteins of approximately 60-68 KDa and 125 KDa were most prominant when RG-2 cells were lysed immediately after removal from culture medium (CM) or after they were incubated with HBSS suppplemented with serum (NCS). RG-2 cells incubated in HBSS containing forskolin (For) or genistein (Gen) had the least amount of protein tyrosyl-phosphorylation, and addition of serum to the incubation medium reduced the effects of forskolin (NCS+For), genistein (NCS+Gen), and genistein and forskolin (NCS+Gen+For) on RG-2 protein tyrosyl-phosphorylation. Strong protein bands were apparent at 44 KDa and 42 KDa when the membrane blots were reprobed for the MAP kinase proteins Erk 1 and Erk 2.

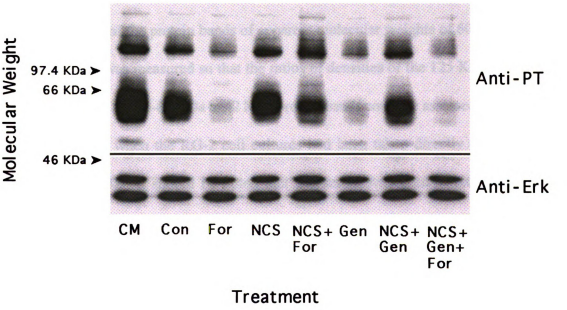

Molecular Weight
97.4 KDa
66 KDa
46 KDa
Anti-PT
Anti-Erk
CM
Con
For
NCS
NCS+ For
Gen
NCS+ Gen
NCS+ Gen+ For
Treatment

Figure 42

protein tyrosyl-phosphorylation was attenuated when serum was present (NCS+For and NCS+Gen) (Figure 42).

The same membrane blots which had been used to visualize tyrosyl-phosphorylation were stripped to remove previous antibodies and were reprobed with an antibody directed against the MAP (mitogen activated protein) kinases Erk 1 (44 KDa) and Erk 2 (42 KDa). Two proteins of apparent molecular weight of 42 KDa and 44 KDa were visualized (Figure 42), and the density of the 42 KDa and 44 KDa protein bands did not appear to vary between treatment groups (Figure 42).

The density of the protein bands of apparent molecular weights of 60-68 KDa, 125 KDa, and 42 KDa was measured so that the ratios of densities of the 125 KDa to 42 KDa protein bands and of the 60-68 KDa to 42 KDa protein bands could be assessed. The density ratios were highest when the RG-2 cell cultures had been taken directly out of culture medium (Figure 43). It should be noted that 2% serum was used in RG-2 studies to compensate for an increased amount of protein and therefore for a potentially increased amount of enzymatic activity. The ratios were lowest when the cultures had been incubated in HBSS supplemented with 5 μM forskolin (For) or 50 μM genistein (Gen) (Figure 43). The 125 KDa/ 42 KDa and 60-68 KDa/42 KDa density ratios for RG-2 cell cultures treated with HBSS containing both 0.5% serum and 5 μM forskolin (NCS+For) were less than those for cultures treated with HBSS containing only 2% serum (NCS) but greater than ratios for cultures incubated with HBSS supplemented with only 5 μM forskolin (For) (Figure 43). Statistical analysis of 3 experiments indicated a significant difference for the 60-68 KDa protein between cultures which were treated with HBSS containing forskolin compared to

Figure 43. Tyrosyl-phosphorylation of 60-68 KDa and 125 KDa proteins from RG-2 cells is demonstrated by densiometric quantification (stellation). RG-2 cells were either lysed immediately after removal of culture medium (CM) or after incubation in HBSS (Con), or in HBSS containing forskolin (For; 5 μM), serum (NCS; 2%), serum and forskolin (NCS+For), genistein (Gen), or serum and genistein (NCS+Gen). The amount of protein tyrosyl-phosphorylation is expressed as the ratio of the density of the phosphorylated proteins of molecular weights 60-68 KDa (solid bars) and 125 KDa (hatched bars) to the density of proteins visualized using the Erk 1 antibody (42 KDa). Cultures incubated in HBSS supplemented with forskolin (For) or genistein (Gen) had the lowest levels of tyrosyl phosphorylation, and those directly out of culture medium (CM) or incubated in HBSS containing serum (NCS) had the highest levels. It should be noted that data for the 60-68 KDa protein could not be obtained in one experiment (A). Results from three experiments (A, B, and C) were pooled, and statistical analysis to determine significant differences between treatment groups was performed. For the 60-68 KDa protein, there were statistical differences between cultures taken directly of of culture medium and those incubated in HBSS alone (CM vs Con), between cultures incubated in HBSS alone and those incubated in HBSS containing 5 μM forskolin (Con vs For), between cultures incubated with HBSS containing forskolin and those incubated with HBSS containing both serum and forskolin (For vs NCS+For), and between cultures incubated in HBSS containing both serum and forskolin and those incubated with HBSS containing serum and forskolin and genistein (NCS+For vs NCS+Gen+For). For the 125 KDa protein, only cultures incubated in HBSS alone compared to those incubated in HBSS containing forskolin (Con vs For) were significantly different from each other. Although several groups were not significantly different from each other, one can see similar trends when individual experiments (A, B, and C) are compared. For example, there appears to be a difference between cultures treated with HBSS supplemented with serum compared to cultures incubated with HBSS containing serum and forskolin (NCS vs NCS+For) and between cultures incubated with HBSS containing only genistein compared to those treated with HBSS containing both serum and genistein (Gen vs NCS+Gen).

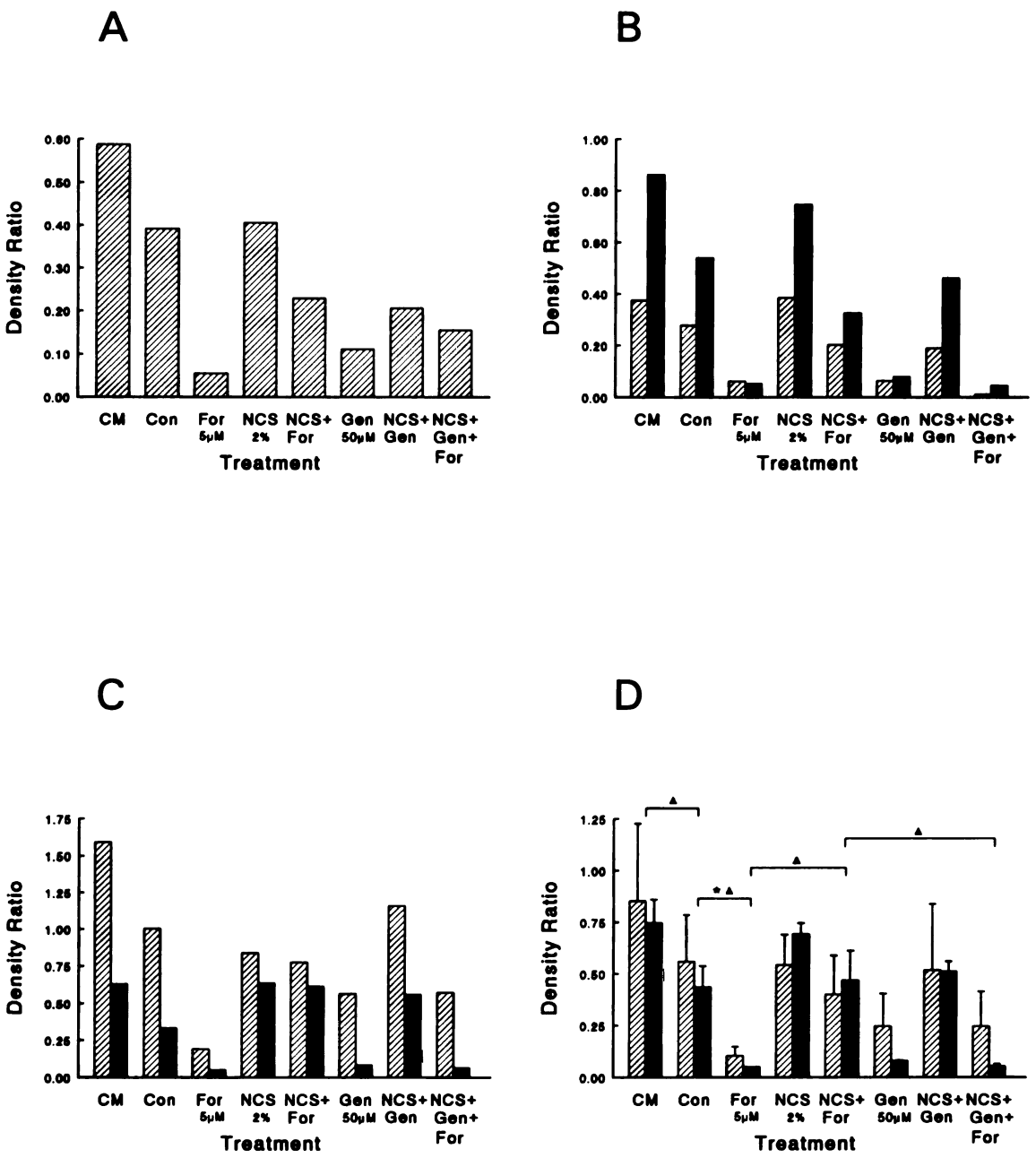

A
B
C
D
Density Ratio
Treatment
CM
Con
For 5μM
NCS 2%
NCS+ For
Gen 50μM
NCS+ Gen
NCS+ Gen+ For

Figure 43

those incubated in HBSS containing both serum and forskolin (Figure 43D). Similarly, density ratios for cultures treated with HBSS containing both 2% serum and 50 μM genistein (NCS+Gen) appeared to be less than those for cultures treated with HBSS supplemented with only 2% serum (NCS) but greater than density ratios for cultures incubated with HBSS containing 50 μM genistein (Gen) only (Figure 43). However, there was no significant difference between these treatment groups when statistical analysis was performed. There was though, a significant difference between cultures incubated in HBSS containing serum and forskolin compared to cultures incubated in HBSS containing serum and forskolin and genistein for the 60-68 KDa protein (Figure 43D).

Cultured RG-2 cells were mostly non-stellate when directly out of culture medium (CM) and when incubated in only HBSS (Con) (Figure 44). A large fraction of RG-2 cells became stellate when incubated for 80 min in HBSS containing 5 μM forskolin (For) (Figure 44). The tyrosine kinase inhibitor, genistein (50 μM), also induced a significant fraction of RG-2 cells to become stellate (Figure 44). RG-2 cells treated with HBSS supplemented with serum (2%) had a significantly smaller fraction of stellate cells compared to controls (Con) (Figure 44). Serum (2%) attenuated forskolin-induced (5 μM) stellation (NCS+For), but this effect of serum on RG-2 morphology was significantly reduced in the presence of genistein (50 μM) (NCS+Gen+For) (Figure 44). Data from these experiments demonstrate that genistein can alone induce stellation and that genistein can attenuate the effects of serum on forskolin-induced stellation. In addition, protein tyrosyl-phosphorylation appears to be high in RG-2 cells exposed to serum but low in RG-2 cells exposed to forskolin or genistein. These results indicate that tyrosine kinase activity is involved in regulating RG-2 cell

Figure 44. RG-2 cell morphology is modulated by cyclic AMP, serum and genistein (stellation). RG-2 cells directly out of culture medium (CM) or incubated in HBSS alone (Con) had a small fraction of stellate cells (< 5%). Incubation of RG-2 cells in HBSS containing serum (NCS) resulted in a significantly smaller fraction of stellate cells compared to those incubated in HBSS alone. HBSS supplemented with forskolin (For) or genistein (Gen) induced a significantly larger fraction of cells to become stellate compared to those incubated in HBSS alone (Con). Inclusion of serum in the modified HBSS solution significantly reduced forskolin (NCS+For) and genistein (NCS+Gen) induced stellation. The fraction of pituicytes which was stellate was larger when cells were treated with HBSS containing serum, genistein, and forskolin (NCS+Gen+For) compared to cultures incubated in HBSS containing only serum and forskolin (NCS+For). (✱ - significantly different from HBSS alone (Con); ▲ - significantly different from each other; ■ - Gen and NCS+Gen are significantly different from each other; $p < 0.05$).

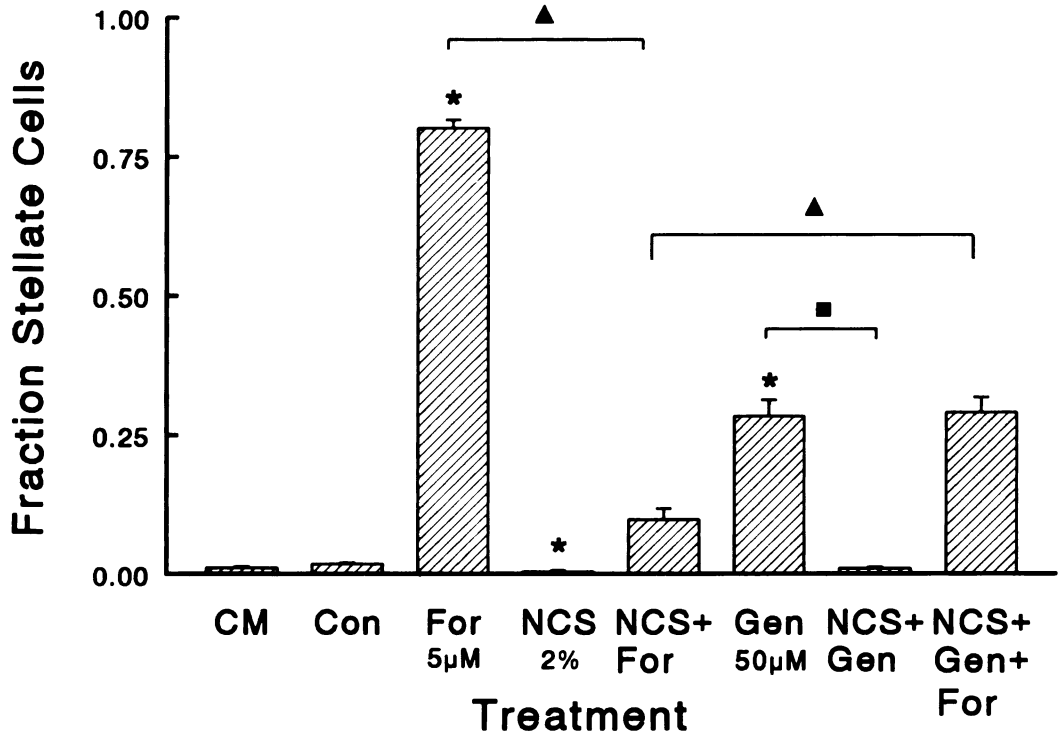

Fraction Stellate Cells
1.00
0.75
0.50
0.25
0.00
CM
Con
For 5μM
NCS 2%
NCS+ For
Gen 50μM
NCS+ Gen
NCS+ Gen+ For
Treatment

Figure 44

morphology and that changes in cell morphology can be associated with changes in protein tyrosyl-phosphorylation.

A subsequent set of RG-2 experiments was performed to determine the effects of tyrosine kinase inhibition on reversal of forskolin-induced stellation by serum and to determine whether there were associated alterations in the tyrosyl-phosphorylation status of presumed cytoskeletal proteins. RG-2 cell cultures were initially incubated in HBSS containing 5 μM forskolin for 60 min to induce stellation and induce changes in protein tyrosyl-phosphorylation. Cultures were then either immediately fixed for morphological evaluation or lysed for Western blot analysis, or they were subsequently incubated in a second experimental medium for 20 min and then evaluated for morphology or prepared for Western Blot analysis.

Tyrosyl-phosphorylation of RG-2 cellular proteins was observed in proteins which were immunoreactive to the phosphotyrosine antibody and visualized using enhanced chemiluminescence (Figure 45). As previously observed, proteins with apparent molecular weights of 60-68 KDa and 125 KDa varied between treatment groups (Figure 45). Protein-tyrosyl-phosphorylation was most prominent in cultures subsequently treated with HBSS supplemented with 2% serum (Figure 45). Although there was also a large amount of protein tyrosyl-phosphorylation in RG-2 cultures subsequently incubated with HBSS containing both 2% serum and 50 μM genistein (For/NCS+Gen), a difference in the relative densities could not be assessed with the naked eye.

Western blot membranes were stripped of the previous antibodies and reprobed with an antibody directed against Erk 1 and Erk 2, and density ratios were assessed for the 125

Figure 45. Tyrosyl-phosphorylation of RG-2 cell proteins is demonstrated by immunocytochemical visualization (destellation). Only a small amount of tyrosyl-phosphorylation of proteins of approximately 60-68 KDa and 125 KDa was observed when RG-2 cells were treated with HBSS containing forskolin and immediatedly lysed (For/out) or treated with HBSS containing forskolin and subsequently treated with HBSS containing genistein (For/Gen). Tyrosyl-phosphorylation of these proteins was most prominent when RG-2 cells previously incubated in HBSS containing forskolin were incubated in HBSS containing serum (For/NCS). The membrane blots were reprobed for the MAP kinase proteins Erk 1 and Erk 2 and proteins of molecular weights of 42 KDa and 44 KDa were visualized.

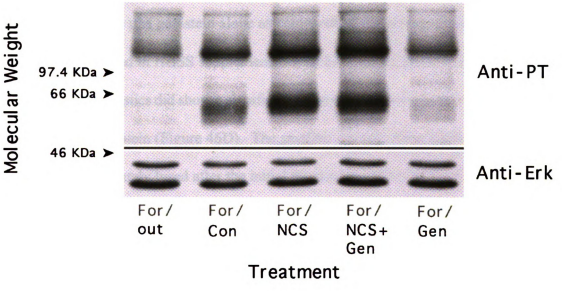

Molecular Weight
97.4 KDa
66 KDa
46 KDa
Anti-PT
Anti-Erk
For/ out
For/ Con
For/ NCS
For/ NCS+ Gen
For/ Gen
Treatment

Figure 45

KDa and 60-68 KDa proteins using the corresponding 42 KDa Erk protein density values. 125 KDa/42 KDa and 60-68 KDa/42 KDa density ratios tended to be greatest when pretreated cultures (5 μM forskolin; 60 min) were subsequently incubated for 20 min in HBSS supplemented with only 2% serum (For/NCS) (Figure 46). There was an apparent reduction in the density ratios when 50 μM genistein was also present in the serum-containing (2%) HBSS solution (For/NCS+Gen) (Figure 46). However, statistical analysis did not show a significant difference between these treatment groups. The relative density ratios were smaller for pretreated cultures which were subsequently incubated with HBSS supplemented with 50 μM genistein alone as compared to pretreated cultures which were subsequently incubated in HBSS supplemented with both 2% serum and 50 μM genistein (For/NCS+Gen). Statistics did show a significant difference between these treatment groups for the 60-68 KDa protein (Figure 46D). The smallest density ratios were observed when cultures were immediately lysed after the initial 60 min incubation in forskolin-containing HBSS (For/out) (Figure 46). There was a significant difference between cultures immediately lysed and cultures which were subsequently incubated in HBSS alone (Figure 46D). Statistical analysis also showed a significant difference between cultures lysed immediately after being incubated in forskolin compared to cultures which were incubated in forskolin and subsequently incubated in serum before being lysed (Figure 46D).

Cultures which were immediately fixed after being incubated in HBSS with forskolin for 60 min had a large fraction of stellate cells, as did pretreated (stellate) RG-2 cells which were subsequently treated with 50 μM genistein for 20 min (Figure 47). Stellate cultures subsequently incubated in HBSS (For/Con) had a relatively smaller fraction of stellate cells

Figure 46. Tyrosyl-phosphorylation of 60-68 KDa and 125 KDa proteins from RG-2 cells is demonstrated by densiometric quantification (destellation). The level of protein tyrosyl-phosphorylation is expressed as the ratio of the density of tyrosyl-phosphorylated proteins of molecular weight 60-68 KDa (solid bars) and 125 KDa (Hatched bars) to the density of proteins visualized with antibody against the 42 KDa MAP kinase protein Erk X. The density ratios were lowest for cultures which had been pretreated for 60 min in HBSS supplemented with 5 μM forskolin and then subsequently treated for 20 min with HBSS supplemented with only forskolin (For/out) or genistein (For/Gen). The density ratios were highest in cultures which had serum included in the second experimental medium (For/NCS and For/NCS+Gen). Statistical analysis only showed significant differences between For/out and For/Con for both proteins and between For/out and For/Con and between For/Gen and For/NCS+Gen for the 60-68 KDa protein (D). However, one can see trends in differences between other treatment groups when comparing individual experiments (A, B, and C).

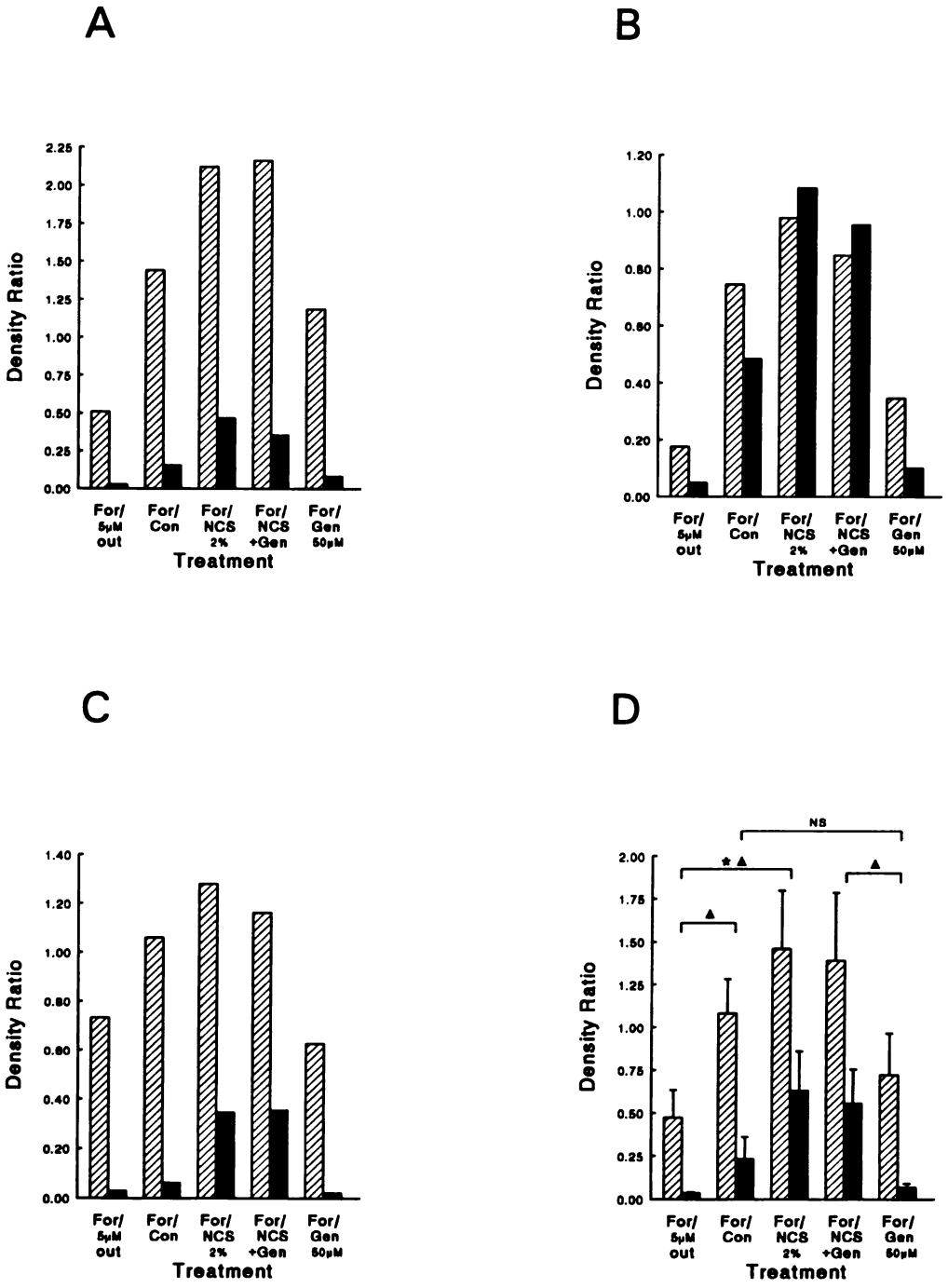

A
B
C
D
Density Ratio
Treatment
For/ 5μM out
For/ Con
For/ NCS 2%
For/ NCS +Gen
For/ Gen 50μM
NS

Figure 46

Figure 47. RG-2 cell morphology is modulated by cyclic AMP, serum, and genistein (destellation). RG-2 cells became stellate when incubated in HBSS containing 5 μM forskolin (For/out). Destellation occured when cultures which had been pretreated with forskolin-containing HBSS for 60 min were subsequently incubated in HBSS (For/Con) or HBSS containing serum (For/NCS) for 20 min, but not when pretreated cells were subsequently incubated with HBSS containing the tyrosine kinase inhibitor genistein (For/Gen). (✱ - significantly different from For/out; ■ - significantly different from For/Con; ▲ - significantly from For/NCS+Gen; $p < 0.05$).

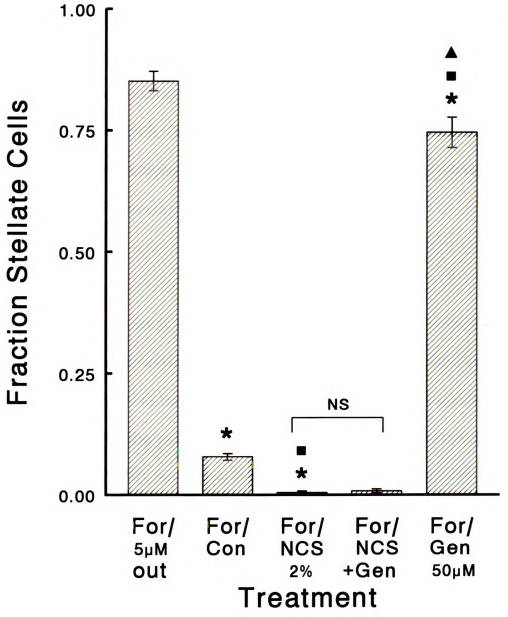

1.00
0.75
0.50
0.25
0.00
Fraction Stellate Cells
NS
For/ 5μM out
For/ Con
For/ NCS 2%
For/ NCS +Gen
For/ Gen 50μM
Treatment

Figure 47

(< 10%) compared to those immediately fixed, and stellate RG-2 cultures which were subsequently treated with HBSS supplemented with either 2% serum (For/NCS) or serum (2%) and genistein (50 μM) (For/NCS+Gen) had a very small fraction stellate cells (Figure 47). Data from these experiments demonstrate that serum reverses forskolin-induced stellation in RG-2 cells and is associated with a relatively larger amount of tyrosyl phosphorylation of the proteins of interest. Treatment of stellate RG-2 cells with genistein caused the cells to remain stellate whereas stellation was reversed when stellate RG-2 cells were incubated in HBSS. These results indicate that tyrosine kinase activity is involved in regulating RG-2 cell morphology and that changes in cell morphology can be associated with changes in protein tyrosyl-phosphorylation.

C. Discussion

Previous studies in the literature have provided indirect evidence indicating that protein tyrosyl-phosphorylation of cytoskeletal proteins is associated with changes in morphology exhibited by cultured cells. Serum and lysophosphatidic acid have been shown to stimulate stress fiber and focal adhesion formation in serum starved Swiss 3T3 cells (Ridley and Hall, 1992). Actin stress fibers are involved in cytoskeletal reorganization, and they are linked to the plasma membrane at focal adhesions, which are cell-extracellular matrix contacts of cells in culture.

Serum and LPA have also been demonstrated to stimulate rapid tyrosyl-phosphorylation of several cellular proteins in serum-starved fibroblasts, including focal adhesion kinase (p125FAK) and paxillin (Seufferlein and Rozengurt, 1994; Schaller and

Parsons, 1994; Barry and Critchley, 1994; Hordijk *et al.*, 1994). It is interesting to note that paxillin is a substrate of activated focal adhesion kinase (Schaller and Parsons, 1995) and that LPA-induced tyrosyl-phosphorylation of focal adhesion kinase has been shown to be paralleled by rapid recruitment of p125FAK, paxillin, talin, vinculin, and PKC-δ to focal adhesion sites (Ridley and Hall, 1994; Barry and Critchley, 1994; Chrzanowska-Wodnicka and Burridge, 1994). However, there are no studies linking fibroblast morphology with protein tyrosyl-phosphorylation.

Protein tyrosine kinases can be broadly divided into two groups: receptor and non-receptor tyrosine kinases. The members of the first class have an extracellular, ligand-binding domain, a transmembrane sequence, and a cytoplasmic domain within which is contained catalytic sequences, and this class of tyrosine kinases functions to transduce a ligand-binding-induced signal into the cytoplasm (Courtneidge, 1994). Non-receptor tyrosine kinases are diverse and include membrane-associated, cytoplasmic, and nuclear proteins. Focal adhesion kinase is a cytoplasmic non-receptor protein tyrosine kinase that is localized at sites of cell adhesion (Courtneidge, 1994).

FAK has been implicated in cellular responses to two distinct classes of extracellular signalling molecules (Hanks, 1995). These include adhesive glycoproteins, such as fibronectin, laminin, and collagen IV, that signal through integrin receptors, and mitogenic neuropeptides, such as bombesin, vasopressin, and endothelin, that signal through G-protein coupled receptors (Zachary and Rozengurt, 1992). FAK has been suggested to be a point of convergence for the action of Src (another tyrosine kinase), peptide growth factors, integrins, and neuropeptides (Courtneidge, 1994). It has been speculated that the function of focal

adhesion kinase is to transmit a signal from integrins and other ligands into the cell; alternatively, it may be responsible for phosphorylating focal adhesion proteins such as tensin, paxillin, and talin, thereby regulating the interaction of focal adhesions with the cytoskeleton.

The studies here were designed to test the hypothesis that the effect of serum on astrocyte morphology is mediated by modulation of tyrosyl-phosphorylation of cytoskeletal proteins. The tyrosine kinase inhibitor genistein was used to see if tyrosine kinase inhibition would alter the effects of serum on pituicyte morphology and tyrosyl-phosphorylation of proteins of interest. Results from these studies provide the first direct evidence that changes in the status of tyrosyl-phosphorylation of cellular proteins in cultured astrocytes is associated with modulation of cellular morphology. Although the 60-68 KDa and 125 KDa proteins expressing treatment-dependent changes in levels of tyrosyl-phosphorylation have not been identified in these studies, the molecular weights of these proteins are consistent with molecular weights of the cytoskeletal proteins paxillin (60-68 KDa) and p125FAK (125 KDa).

The selective tyrosine kinase inhibitor genistein (Akiyama *et al.*, 1987) has been shown to block serum-induced tyrosyl-phosphorylation of focal adhesion kinase and assembly of stress fibers in Swiss 3T3 cells (Chrzanowska-Wodnicka and Burridge, 1994). Genistein was used in experiments presented here to investigate if tyrosine kinase inhibition would prevent serum from attenuating and reversing forskolin-induced stellation in cultured pituicytes. Genistein significantly reduced the effects of serum to attenuate and somewhat reverse forskolin-induced stellation, indicating that tyrosine kinase activity is required for

serum to modulate pituicyte morphology.

Since the amount of protein material obtained from pituicyte cultures was insufficient for Western blot analysis, RG-2 cells were used as a model to investigate astroglial morphology and associated protein tyrosyl-phosphorylation. Western blot analysis revealed that RG-2 cells incubated in HBSS supplemented with serum or taken directly out of culture medium (which contains 10% serum) exhibited strong tyrosyl-phosphorylation of proteins of molecular weights of 60-68 KDa and 125 KDa. Tyrosyl-phosphorylation of these proteins was decreased when cells were incubated in serum-free HBSS and this reduction in tyrosyl-phosphorylation was greatly enhanced by addition of forskolin or genistein to the serum-free HBSS solution. RG-2 cell cultures which had high levels of tyrosyl-phosphorylation of 60-68 KDa and 125 KDa proteins were mostly non-stellate. Cultures incubated in HBSS containing genistein or forskolin not only contained cells with low levels of tyrosyl-phosphorylation of the 60-68 KDa and 125 KDa proteins, but also had a significantly increased fraction of stellate cells. These results indicate that increased intracellular cAMP concentration can mediate stellation and reduce tyrosyl-phosphorylation of the 60-68 KDa and 125 KDa proteins. Whether an increase in intracellular cGMP concentration has the same effect as an increase in intracellular cAMP concentration on the tyrosyl-phosphorylation status of the two proteins of interest is currently unknown and should be investigated in future studies. When serum was included in HBSS containing either forskolin or genistein, an increased amount of tyrosyl-phosphorylation of the proteins of interest was observed, as was a corresponding decrease in the amount of stellation. Addition of genistein to HBSS containing both serum and forskolin resulted in both an increase in

stellation and a decrease in tyrosyl-phosphorylation of the two proteins of interest compared to HBSS containing only serum and forskolin. These results not only demonstrate an association between tyrosyl-phosphorylation of the 60-68 KDa and 125 KDa proteins and cell morphology, but also indicate that serum mediates its effects on cell morphology through activation of a tyrosine kinase.

It is important to note that RG-2 cells treated with HBSS containing only genistein had a significantly larger fraction of stellate cells compared to those incubated in only HBSS. This indicates that there is a tonically active tyrosine kinase in these cells and that the activity of this kinase is required to maintain RG-2 cells in a non-stellate form. Destellation experiments support this hypothesis, since removal of forskolin and subsequent incubation in HBSS alone was enough to cause RG-2 cells to revert back to a non-stellate morphology and for tyrosyl-phosphorylation of the 60-68 KDa and 125 KDa proteins to return to increased levels. The fact that genistein alone did not induce stellation in cultured pituicytes could be explained by a difference in the amount of intrinsic tyrosine kinase activity in these two cell types. The hypothesis that pituicytes have a lower level of intrinsic tyrosine kinase activity is supported by the finding that removal of the stimulus (forskolin) alone is not enough to cause reversion of pituicytes from a stellate to a non-stellate form.

That RG-2 cells incubated in HBSS containing forskolin had a stellate morphology and an associated low level of tyrosyl-phosphorylation of the 60-68 KDa and 125 KDa proteins indicates that forskolin induces stellation via a reduction of tyrosyl-phosphorylation of these proteins. This reduction of protein tyrosyl-phosphorylation may be achieved by either a decrease in tyrosine kinase activity or an increase in phosphatase activity. The

findings in these studies support the hypothesis that stellation of RG-2 cells by forskolin is induced through a cAMP-dependent decrease in the activity of a tonically active tyrosine kinase.

Fibroblast studies in the literature indicate that LPA-induced tyrosyl-phosphorylation is dependent on the small GTP-binding protein Rho. Since LPA-mediated p125FAK phosphorylation has been shown to be inhibited by the *Botulinum* C3 exoenzyme (Kumagai *et al.*, 1993) which also inactivates RhoA, it has been suggested that p125FAK is phosphorylated downstream of RhoA activation (Craig and Johnson, 1996). Also, microinjection of active Rho mimics exogenous LPA and results in recruitment of tyrosyl-phosphorylated proteins to focal adhesion sites followed by focal adhesion assembly and stress fiber formation (Ridley and Hall, 1992; Ridley and Hall, 1994; Barry and Critchley, 1994). If serum regulates cytoskeletal elements in astroglial cells in a manner which is analogous to that observed in fibroblasts, then it is possible that the small GTP-binding protein RhoA may be involved in regulating astroglial morphology.

Recent studies have also indicated that LPA receptors are coupled to Gi and Gq proteins, where the Gi protein mediates a pertussis toxin sensitive mitogenic pathway and the Gq protein mediates a pertussis toxin insensitive pathway involved in focal adhesion formation and stress fiber assembly (Craig and Johnson, 1996). U73122, an inhibitor of LPA-stimulated phospholipase C (PLC) activity, has been shown to block focal adhesion and stress fiber assembly (Chrzanwska-Wodnicka and Burridge, (1994) in fibroblasts, and it has been suggested that Gq activation of PLCβ (the PLC isoform which is activated by G-proteins) is part of the signalling pathway leading to LPA activation of RhoA (Craig and

Johnson, 1996). Previous pituicyte experiments have demonstrated that serum regulates pituicyte morphology in a pertussis toxin-insensitive manner. This finding would therefore support the hypothesis that serum acts via a Gq protein and increases PLCβ activity to mediate its effects on astroglial morphology.

Interestingly, Zachary *et al.* (1992) have demonstrated that bombesin, endothelin, and vasopressin stimulate rapid tyrosyl-phosphorylation of several cellular proteins including focal adhesion kinase and paxillin in serum-starved fibroblasts. Bombesin has been demonstrated to be involved in reorganization of the fibroblast actin cytoskeleton (Ridley and Hall, 1992), and endothelin has been shown to reorganize actin stress fibers and to reverse cAMP-mediated stellation of cortical cultured astrocytes (Koyama *et al.*, 1993; Koyama and Baba, 1994). It is possible that vasopressin is also involved in regulation of cell morphology, although this has never been demonstrated. If vasopressin induces tyrosyl-phosphorylation of cytoskeletal proteins in pituicytes *in vivo*, it could contribute to modulation of pituicyte ultrastructure in the neurohypophysis. Vasopressin could therefore potentially regulate its own release by modulating pituicyte ultrastructure via tyrosyl-phosphorylation of cytoskeletal elements. These assertions that vasopressin may be involved in regulating pituicyte morphology or involved in altering the tyrosyl-phosphorylation status of cytoskeletal proteins are completely speculative and will require further investigation.

The studies presented here have revealed an association in RG-2 cells between the degree of stellation and the status of protein tyrosyl-phosphorylation, particularly of what are presumed to be the cytoskeletal proteins p125FAK and paxillin. Pituicyte studies presented previously have shown that serum attenuates forskolin-induced stellation and reverses

Figure 48. Depiction of possible biochemical pathways mediating the effects of serum on astroglial cell morphology. Maintenance of a non-stellate astroglial morphology may require activation of RhoA, tyrosyl-phosphorylation of focal adhesion kinase, and recruitment of focal adhesion proteins and stress fiber assembly. Increased intracellular cAMP concentration may decrease protein tyrosine kinase activity or increase protein phosphatase activity to inhibit protein tyrosyl-phosphorylation of cytoskeletal proteins, which would prevent recruitment of focal adhesion proteins and stress fiber assembly and therefore cause an instability in the actin cytoskeleton to induce stellation.

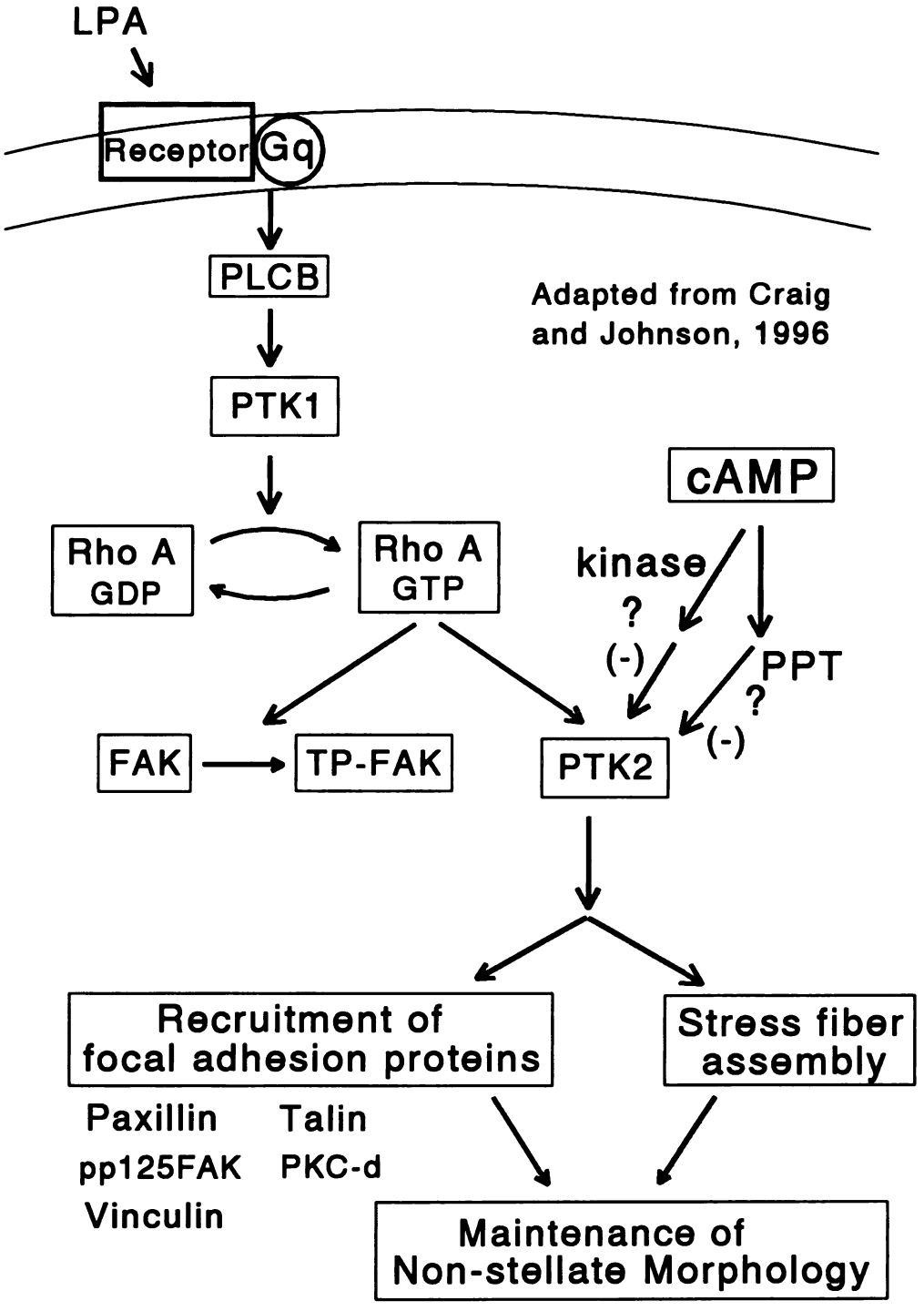

LPA
Receptor
Gq
PLCB
PTK1
Adapted from Craig and Johnson, 1996
cAMP
Rho A GDP
Rho A GTP
kinase
?
(-)
PPT
?
(-)
FAK
TP-FAK
PTK2
Recruitment of focal adhesion proteins
Stress fiber assembly
Paxillin
Talin
pp125FAK
PKC-d
Vinculin
Maintenance of Non-stellate Morphology

Figure 48

stellation in a manner which is pertussis toxin insensitive. These findings indicate that astroglial morphology may be modulated by serum via a pathway similar to what has been proposed for LPA in modulation of the fibroblast cytoskeleton. It is proposed here that tyrosyl-phosphorylation of focal adhesion kinase and paxillin are required to maintain astroglial cells in a non-stellate form and that an increase in intracellular cAMP concentration decreases tyrosine kinase activity or increases protein tyrosine phosphatase activity to cause a reduction in tyrosyl-phosphorylation of these cytoskeletal proteins and therefore an instability in the cytoskeleton which leads to a stellate morphology. A depiction of this proposed mechanism is shown in Figure 48. Further studies will be required to confirm the validity of this proposed pathway, and immunoprecipitation experiments should be performed to confirm that the 125 KDa and 60-68 KDa proteins of interest are truely focal adhesion kinase and paxillin respectively.

CONCLUSIONS

Results from the studies presented here have demonstrated that: 1) agents which increase intracellular cAMP or cGMP concentration can induce stellation of cultured pituicytes; 2) serum attenuates and reverses forskolin-induced stellation of pituicytes in culture, and serum appears to exert its effects on pituicyte morphology by acting downstream of changes in intracellular cAMP concentration; 3) changes in pituicyte morphology occur independently of alterations in intracellular calcium concentration; and 4) changes in the tyrosyl-phosphorylation of 60-68 KDa and 125 KDa proteins are associated with changes in astrocyte morphology.

The main focus of the present studies has been to examine signal transduction mechanisms which mediate morphological plasticity of astrocytes. Pituicytes, astrocytes derived from adult rat neurohypophyses, and RG-2 cells, a representative astroglial cell line, have been utilized to explore these biochemical mechanisms. In addition, effects of several potential mediators of pituicyte ultrastructural changes *in vivo* have been examined on pituicyte morphology *in vitro* in hopes of elucidating substances which modulate pituictye ultrastructure and regulate HNS hormone secretion.

It appears that there are several possible substances which could influence pituicyte ultrastructure *in vivo*. Although oxytocin and vasopressin would seem like obvious potential

modulators of pituicyte ultrastructure *in vivo*, and V_1 vasopressin receptors have been identified on pituicytes (Hatton *et al.*, 1992), no studies have demonstrated that oxytocin and vasopressin affect pituicyte morphology. However, there is growing evidence from studies on fibroblasts which suggest that vasopressin may serve to maintain pituicytes in a non-stellate or unretracted form, and future pituicyte studies should address this question.

Since the neurohypophysis is innervated by noradrenergic fibers and norepinephrine can access neurohypophysial pituicytes directly from the circulation due to the absence of a blood brain barrier, there is the potential for norepinephrine to alter intracellular cAMP concentration and affect pituicyte ultrastructure *in vivo*. The fact that systemic levels of epinephrine increase during stress and with other conditions associated with activation of the HNS (Holzbauer *et al.*, 1980), and the demonstration that intraperitoneal injections of hypertonic saline do not induce withdrawal of pituicyte cytoplasmic material in the neurohypophysis in adrenal medullectomized rats (Beagley and Hatton, 1994), also suggests a possible role for circulating epinephrine and increased intracellular cAMP concentration to regulate pituicyte ultrastructure *in vivo*. Lactation and saline-induced dehydration has also been associated with elevated levels of intracellular cAMP within the neurohypophysis (Ruoff *et al.*, 1976), further indicating that cAMP is involved in regulating pituicyte ultrastructural plasticity.

Although norepinephrine has been demonstrated to induce stellation of cultured pituicytes (Bicknell *et al.*, 1989 and Hatton *et al.*, 1991), the mechanism mediating noradrenergic-induced stellation of pituicytes in culture had not previously been investigated. The studies presented here demonstrate that forskolin, which activates adenylate cyclase

directly, and the cAMP analog 8-bromo cAMP induce stellation of cultured pituicytes, indicating that increased intracellular cAMP concentration mediates stellation of pituicytes in culture. Although these agents may increase intracellular cAMP concentration to cause activation of cAMP-dependent protein kinase, which then phosphorylates a protein, such as a phosphatase, which eventually results in a change in cytoskeletal components that leads to stellation of cultured pituicytes, results from PKA-inhibitor experiments do not support this hypothesis. The paradoxical effects observed with the protein kinase A inhibitors are difficult to interpret, although one explanation for their effects on pituicyte morphology is that they bind to and mediate their effects through the regulatory subunit of protein kinase A. It may be advantageous to further investigate how the protein kinase inhibitors are mediating their effects on pituicyte morphology.

In addition to localization of nitric oxide synthase in the magnocellular axons projecting to the neurohypophysis (Bredt *et al.*, 1990 and Dawson *et al.*, 1991) and in the neurohypophysis itself (Sagar and Ferriero, 1987), NOS and NOS mRNA have been shown to increase when the HNS is activated during lactation and during dehydration (Ceccatelli and Eriksson, 1993; Villar *et al.*, 1994; Sagar and Ferriero, 1987). These findings indicate that there is the potential for nitric oxide and therefore increased intracellular cGMP to influence pituicyte ultrastructural plasticity *in vivo*. Another indication that intracellular cGMP could potentially modulate pituicyte ultrastructure is the fact that atrial natriuretic peptide has been localized in the neurohypophysis and that ANP receptors are present on pituicytes (Gutkowska *et al.*, 1987).

Nitric oxide donors, atrial natriuretic peptide, and 8-bromo cGMP have been

demonstrated in the present studies to induce stellation of cultured pituicytes. Although in many systems cGMP activates protein kinase G to mediate its effects, further investigations will be required to determine if this is occurring in cultured pituicytes. One possible study to investigate if inhibition of protein kinase G can inhibit cGMP mediated stellation would be to treat cultured pituicytes with a protein kinase G selective protein kinase inhibitor, such as HA-1004 in addition to a substance which increases intracellular cGMP concentration. Whether increased intracellular cGMP concentration in pituicytes is involved in cross-talk with the cAMP biochemical pathway which mediates stellation of culture pituicytes should also be further investigated.

Since the blood brain barrier is absent in the neurohypophysis, components in blood serum should be considered as potential sources of influence on pituicyte ultrastructure. There are several lines of evidence indicating that serum may modulate pituicyte ultrastructural plasticity. It is known that pituicytes are exposed to serum *in vivo* and that ultrastructural changes in the neurohypophysis are reversible (Tweedle and Hatton, 1980a). Also, retraction of pituicyte cytoplasmic material *in vivo* (serum is present) takes substantially longer than stellation of pituicytes *in vitro* (in serum-free media). Bicknell *et al.* (1989) observed that withdrawal of serum induced some cultured pituicytes to become stellate, which may have been the first indication that serum maintains pituicytes in a non-stellate morphology. It is feasible to speculate that a substance which is stimulating withdrawal of pituicyte cytoplasmic material *in vivo*, such as norepinephrine, epinephrine, nitric oxide and/or ANP, must reach a particular concentration in the pituicyte microenvironment to in effect overcome the effects of serum and therefore cause retraction

of pituicyte membrane and cytoplasm.

As we and others have observed, pituicytes and other cultured astroglial cells are non-stellate when incubated in normal growth medium. Normal growth medium used in pituitcyte studies contains 10% newborn calf serum. Studies investigating the effects of serum on C-6 glioma cells (Tas and Koschel, 1990; Koschel and Tas, 1993) demonstrated that serum can reverse cAMP mediated stellation of these cells, although the biochemical mechanism mediating the effects of serum on cell morphology is currently unknown. Studies presented here have demonstrated that serum attenuates and reverses cAMP mediated stellation in cultured pituicytes, and that serum does not exert its effects on cAMP mediated stellation by acting via an inhibitory G-protein nor by increasing phosphodiesterase activity, but rather by acting downstream of changes in intracellular cAMP concentration. It would be interesting to perform additional studies to investigate if serum can also attenuate and reverse cGMP mediated stellation in cultured pituicytes.

Serum has been demonstrated here to regulate maintenance of cultured pituicytes in a non-stellate form. Studies performed by other investigators have indirectly indicated that serum is involved in maintaining pituicytes in a form where the cytoplasmic material is interposed between axon terminals and between axon terminals and the basal lamina *in vivo*. One may speculate that *in vivo*, serum is involved in maintaining the pituicyte barrier and therefore prevents hormone release into the systemic circulation until the HNS is activated and release of hormone into the sytemic cirulation is required.

There has been conflicting evidence in the literature as to whether or not Ca^{+2} plays a role in mediating alterations in astroglial morphology. In addition, the role of Ca^{+2} in

mediating stellation or destellation in the same cell type has not been previously investigated. Whether or not increased intracellular Ca^{+2} concentration is required for forskolin-induced stellation or serum-induced destellation has therefore been investigated in cultured pituicytes. The studies presented here have demonstrated that forskolin- and serum-induced changes in pituicyte morphology occur even in the absence of calcium, and it has therefore been concluded that morphological alterations in cultured pituicytes occur independently of changes in intracellular Ca^{+2} concentration.

The studies presented here indicate that inhibition of tyrosine kinase activity by genistein attenuates the effects of serum to prevent forskolin-induced stellation and to cause destellation in cultured astroglial cells. Two findings from these studies must be carefully considered: 1) genistein alone induces stellation of RG-2 cells but not pituicytes and 2) incubation in HBSS causes stellate RG-2 cells but not stellate pituicytes to revert to a non-stellate form. These results indicate that RG-2 cells have a much higher intrinsic tyrosine kinase activity than pituicytes. If different cell types adapt particular features to improve their specific function or viabiltiy, one can speculate as to why two cell types from different areas of the brain would have different levels of intrinsic tyrosine kinase activity.

If the level of tyrosine kinase activity is involved in determining astroglial ultrastructure *in vivo*, it is possible that pituicytes have adapted a low intrinsic tyrosine kinase activity so that once they are stimulated to withdrawal their cytoplasmic material from between the axon terminals and from between the axon terminals and the basal lamina that they will be less likely to spontaneously reinsert their cytoplasmic material and reform a physical barrier, which could potentially interfere with normal hormone release from the

neurohypophysis into the systemic circulation. It is then plausable that when activation of the HNS has ceased and the concentration of the stimulating substance is reduced, that serum in the systemic circulation can then overcome the stimulating substance and induce pituicytes to reinsert their cytoplasmic interpositions.

One may then speculate that astrocytes from brain regions which have a blood brain barrier do not have access to serum, and therefore since there is no serum present to enhance cellular tyrosine kinase activity, these cells must have a high intrinsic level of tyrosine kinase activity to achieve a form which is analogous to non-stellate *in vitro*. If these speculations are correct, then it may be hypothesized that intrinsic levels of tyrosine kinase activity regulate the maintenance of an ultrastructural form *in vivo* that is equivalent to the non-stellate form *in vitro*, which therefore regulates physical barriers to modulate secretion of hormones, growth factors, neurotransmitters, or other substances.

Additional results from the RG-2 cell studies have demonstrated an association between astroglial cell morphology and tyrosyl-phosphorylation of the presumed cytoskeletal proteins focal adhesion kinase and paxillin. The data from these studies may provide the first insight as to how cAMP and serum may regulate astroglial morphology. It may be possible to analyze astrocytes from different brain regions and determine their intrinsic tyrosine kinase activites. This information could enhance our understanding of the regulation of hormone or neurotransmitter secretion from different areas of the brain. As plasticity occurs throughout the brain, pituicytes, which are plastic *in vivo* as well as *in vitro* serve as a model for studying brain plasticity in general. Although RG-2 cells are an astroglial cell line, they too appear to function as a model to study plastic events which occur in the brain. These

studies may have serious implications in not only providing a better understanding of astrocyte plasticity and regulation of hormone and neurotransmitter secretion, but may also pave the the way to a better understanding and improved treatment of diseases which are due to astroglial abnormalities.

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