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Participation of the Autonomic Nervous System in the Beta-Adrenergic Agonist-Induced Expression of the Cystatin S Gene in Rat Submandibular Glands

By

Orlando Chaparro

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The city University of New York.

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ABSTRACT

The autonomic nervous system regulates the volume, rate of secretion and composition of saliva. Parasympathetic innervation of rat submandibular glands is present at birth; sympathetic innervation only reaches the gland by postnatal day 5. Isoproterenol (IPR), a β-adrenergic agonist, induces hypertrophic and hyperplastic enlargement of rat salivary glands and expression of a number of genes including cystatin S, a member of family 2 of the cysteine proteinase inhibitor superfamily. Cystatin S gene expression is tissue and cell type specific, temporally regulated during postnatal development, and not observed in adult animals unless stimulated by IPR. Enlargement of submandibular glands and cystatin S gene expression can also be induced by continuous amputation of the lower incisor teeth or intraoral administration of the cysteine protease papain. It has been suggested that these responses are mediated by the autonomic nervous system. Experiments in this study were designed to analyze the participation of the autonomic nervous system in the IPR-induced expression of the cystatin S gene.

Sympathetic denervation (bilateral and unilateral) achieved by removing the superior cervical ganglion reduced the IPR-induced expression of the cystatin S gene. Chemical sympathectomy produced by treatment of the rats with reserpine resulted in reduction of expression of the gene comparable to that produced by

surgical sympathectomy. Bilateral parasympathectomy (by severing the chorda tympani/lingual nerve) also reduced IPR-induced expression of cystatin S gene. Unilateral parasympathectomy or unilateral glossopharyngeal denervation in contrast, had no effect on IPR-induced cystatin S gene expression. Cystatin S gene expression was induced by IPR during early postnatal development before the sympathetic nerve terminals reach the gland by 5 days of age. The levels of cystatin S mRNA remained constant until day 8, when a substantial increase in IPR-induced expression of the gene was observed. This increased expression in response to IPR was reduced by sympathectomy of one day-old animals. Collectively, these data suggest a role of the autonomic nervous system in the regulation of the cystatin S gene expression and indicate that factor(s) from the sympathetic and the parasympathetic nerve terminals participate in the IPR-induced expression of the cystatin S gene.

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CHAPTER I

HISTORICAL REVIEW AND BACKGROUND

1. PROTEOLYSIS AND PROTEASES

Proteases are enzymes that play a key role in protein metabolism. They participate in the regulation of many cellular and extracellular processes, and different proteolytic events take place in each of the functionally distinct intracellular compartments. In order to prevent tissue damage or abnormalities in cell metabolism, the activity of proteolytic enzymes needs to be carefully regulated (Twining, 1994). Proteolytic activity can take the form of the precise cleavage of a single peptide bond that modifies or switches on or off the biological activity of a target protein. On the other hand, all the polypeptide bonds in a protein substrate may be hydrolyzed to regulate its intracellular level and to release free amino acids that can be recycled into new proteins. The extent of the proteolytic process depends on both the specificity of the participating proteases and the environment that the cell provides for their action (Barrett, 1992).

Proteases serve a variety of physiological functions at the cellular, tissue and systemic level and in many pathological events. For example, specific

intracellular endopeptidases cleave the signal peptide found in numerous proteins in prokaryotic and eukaryotic cells, and the polypeptides that need to be removed in order to activate many prohormones, cytokines and enzymes (Hazuda *et al.*, 1990). Lysosomal and ubiquitin-mediated intracellular protein turnover is carried out by proteases that participate in the control and selectivity of the degradation process (Finley and Chau, 1991; Rivett, 1993), whereas extracellular proteases mediate the turnover of extracellular matrix proteins, a critical process that regulates the life time of molecules responsible for cellular and extracellular functions (Woessener, 1991). It has been suggested that proteases are involved in the regulation of the cAMP signaling pathway (Ebina *et al.*, 1997), and that some proteases, like calpain, may act as mediators in signal transduction pathways (Rosser *et al.*, 1993). Proteases also modulate cell growth (Scott, 1992) and are involved in tissue remodeling associated with normal growth and development (Matrisian and Hogan, 1990; Werb *et al.*, 1992). Neuronal growth (Cunningham, 1992), angiogenesis (Niedbala, 1993), and normal (Scott, 1992) and malignant cell proliferation (Scott and Tse, 1992), all require the involvement of proteolytic processes.

Tissues need to be able to control not only endogenous proteases, but also exogenous proteases released by microorganisms, inflammatory cells, and tumor cells. These microorganisms and cells release proteases that facilitate their invasion into tissues. Monocytes, macrophages, polymorphonuclear leukocytes,

mast cells, and natural killer cells can release proteases that are targeted toward killing invading organisms, as well as cells (Evans, 1991; Katunuma, 1990; Twining, 1994).

Living organisms have a remarkable capacity to degrade their own proteins and the control of proteolysis in tissues requires the regulation of multiple proteases involved in any given degradative process. Metastasis of tumor cells, for example, involves several proteases including cathepsins B, L and D, plasminogen activators, and matrix metalloproteases, depending on the tumor type or oncogenic transformation (Rechberger and Woessner, 1993; Stracke and Liotta, 1992). Degradation of joints in arthritis also involves numerous proteases (Hembry *et al.*, 1993).

There are multiple mechanisms regulating proteolytic activity in cells and the extracellular matrix of tissues. The mechanisms controlling a given protease depend upon the protease itself, its particular form (zymogen, active form, or modified form), and its location within a specific cell compartment or within the tissue. Frequently, proteases, including lysosomal proteases, are synthesized as inactive zymogen molecules that are activated by a number of modifications including carbohydrate restructuring and/or proteolytic cleavage, that may require amplification systems in which several zymogens are activated sequentially, culminating in the activation of a protease that plays a major role in a given physiological process {e.g., the coagulation pathway (Mann *et al.*, 1988)}. The

catalytic activity of mature proteases is controlled by pH, post-translational modifications (phosphorylation, glycosylation, or oxidation), localization (secretory granules, lysosomes, or membranes), reaction with activators or inhibitors, and proteolytic degradation. (reviewed in Twining, 1994).

Proteases are either *endopeptidases* that cleave polypeptide chains in their inner regions, or *exopeptidases* that cleave near an N- or C-terminus. O*ligopeptidases* comprise a group of endopeptidases that act only on substrates smaller than proteins, typically up to 20-30 amino acid residues (Barrett, 1992). Four major classes of endopeptidases are responsible for proteolytic activity in eukaryotes. They are classified on the basis of the chemical groups responsible for their catalytic activity: *serine, cysteine, aspartic,* and *metalloproteases* (Barrett, 1992; Twining, 1994).

2. CYSTEINE PROTEASES

Cysteine proteases are proteolytic enzymes that have a cysteine residue at the catalytic site (Barrett, 1987; Bobek and Levine, 1992; Turk *et al.*, 1986). There are two major groups of cysteine proteases: the *lysosomal proteases* (cathepsins B, H, L, and S) and the *cytosolic calpains* I and II (Twining, 1994). Calpains are a special group of cysteine proteases that require calcium for activity (Saido *et al.*, 1994; Suzuki *et al.*, 1992).

Cysteine proteases include endopeptidases (lysosomal cathepsins B, H, and L, for example). Endopeptidases, released from lysosomes in various pathological processes such as inflammation and tumor invasion, play a major role in cellular protein turnover. Cysteine proteases also include exopeptidases like cathepsin C (dipeptidyl peptidase I), which is also found in lysosomes (Bobek and Levine, 1992).

Several normal processes involve the activity of cysteine proteases: intracellular catabolism of proteins and peptides (Barrett and Kirschke, 1981), processing of proenzymes and prohormones (Marks *et al.*, 1986), breakdown of collagen (Etherington, 1980; Turk *et al.*, 1997), bone resorption (Delaisse *et al.*, 1984) (Delaisse *et al.*, 1984), and apoptosis (Fernandes-Alnemri *et al.*, 1995; Martins and Earnshaw, 1997). Cystein proteases play a role in modulating the penetration and destruction of tissues during infection (Barrett *et al.*, 1984; Björck *et al.*, 1989). Proteolytic processing by cysteine proteases has also been shown to regulate the assembly of many viral proteins (Korant *et al.*, 1988), including HIV-I (Guy *et al.*, 1991).

Increased levels of cysteine proteases have been observed in several pathophysiological conditions. For example, the synovial cells that are attached to cartilage and bone at sites affected by rheumatoid joint erosion, display an enhanced transcription of the cathepsin B gene when compared to normal fibroblasts (Trabandt *et al.*, 1991). Cysteine proteases can also be released from

cells into the extracellular matrix and participate in the extracellular degradation of the gum tissue associated with periodontal disease (Lah *et al.*, 1993). Many tumor cells also abnormally synthesize and secrete cathepsins B and L (Qian *et al.*, 1991).

Active proteases are carefully regulated. These enzymes can be modified by phosphorylation and glycosylation, stored in vesicles, and/or localized in membranes. Their activity can also be controlled by pH, calcium ions, ATP, and by specific cysteine protease inhibitors (reviewed in Twining, 1994).

3. CYSTEINE PROTEASE INHIBITORS: CYSTATINS

The activities of cysteine proteases are controlled by inhibitory proteins such as α_2-macroglobulin and cystatins. α_2-macroglobulin is an unusual inhibitor of proteases in that it reacts with most proteases of all four major classes (Borth, 1992). The only known mammalian inhibitor of aspartic proteases is α_2-macroglobulin (Thomas *et al.*, 1989). The activity of lysosomal cysteine proteases, cathepsins B and H toward protein and peptide substrates at pH 6.2 is also blocked by α_2-macroglobulin (Mason, 1989).

Cystatins are naturally occurring cysteine protease inhibitors. These inhibitors protect cells from inappropriate endogenous or exogenous proteolysis and/or may be involved in the control mechanisms responsible for intracellular or extracellular protein breakdown. Cystatins are inhibitors that bind tightly and

reversibly to endogenous or exogenous cysteine proteases of bacterial, and viral origin (Bobek and Levine, 1992). All known cystatins belong to an evolutionarily-related superfamily composed of three major families, **stefins** (family 1), **cystatins** (family 2), and **kininogens** (family 3). Members of family 1, the stefins, are found primarily intracellularly, contain about 100 amino acid residues (~11 KDa) and lack disulfide bonds. The human cystatins A and B, as well as the rat cystatins α and β, are representatives of this family. Members of family 2, cystatins, are found primarily in body fluids but can also be found in tissues. They contain about 120 amino acid residues (~14 KDa), and two intra-chain disulfide bonds. The chicken egg white cystatin, cystatin C, and salivary cystatins, including rat cystatin S, belong to this family. Family 3 members, the kininogens, are found in the plasma and secretions of mammalian species. Three major types of kininogens, the low-molecular weight (LMWK), high-molecular weight (HMWK), and the T-kininogens are single-chain glycoproteins with a molecular weight of ~50-120 KDa. They are comprised of three cystatin-like domains that are thought to be the result of gene duplication during evolution. The kininogens contain additional disulfide bonds and are glycosylated (Barrett, 1986; Bobek and Levine, 1992; Rawlings and Barrett, 1990).

The concept of a "cystatin superfamily" emerged from the observations that multiple cystatin-like sequences are present in the kininogens and that stefins are related to both the cystatins and the repeats in the kininogens (Ohkubo *et al.*,

1984). The cystatin superfamily has been dramatically enlarged in recent years. Several new members have been characterized, including proteins from insects and plants (Brown and Dziegielewska, 1997). In addition to the original stefin, cystatin and kininogen families, it is clear that the **fetuins** (Elzanowski *et al.*, 1988), **histidine-rich glycoproteins (HRG)** (Koide and Odani, 1987), and the **cystatin-related proteins (CRP)** (Devos *et al.*, 1993; Devos *et al.*, 1995), represent new families of proteins within the cystatin superfamily. Some of these proteins are active cysteine protease inhibitors, while others have lost or perhaps never acquired this inhibitory activity. The fetuins, HRGs, CRPs and domain 1 of the kininogens have all lost some of the amino acids that are known to be important for inhibition of cysteine protease activity (Brown and Dziegielewska, 1997).

Based on partial amino acid homology, new members, such as the invariant chain (Ii), a non-polymorphic protein intimately involved in the assembly of class II MHC molecules, and the transforming growth factor-β receptor type II (TGF-β receptor II), may in fact, represent members of another emerging family within the superfamily that may have used some common building blocks to form functionally diverse proteins (Brown and Dziegielewska, 1997; Katunuma *et al.*, 1994). Proteins containing repeated structures are, of course, not unique to the cystatin superfamily; however, the cystatin superfamily is possibly unique in that so many members have survived through evolution and are still currently represented (Brown and Dziegielewska, 1997).

An abnormal balance between cysteine proteases and their inhibitors has been observed in several pathological conditions, such as inflammation (Jarvinen *et al.*, 1987; Mort *et al.*, 1984) and malignancy (Sloane, 1990). Levels of cystatin C in various body fluids have been used as an indicator of disease. For example, the concentration of cystatin C in urine of normal subjects is about 0.1 μg/ml, whereas in patients with renal tubular disorders the level rises about 200-fold (Lofberg and Grubb, 1979). Additionally, sera of patients suffering from the autoimmune diseases lupus erythematosus and glomerulonephritis were found to contain about ten times higher concentration of cystatin C than those of control patients (Brzin *et al.*, 1984).

Hereditary cystatin C amyloid angiopathy (HCCAA) was the first human genetic disorder identified that is caused by an abnormal cysteine protease inhibitor. This disease is transmitted as an autosomal dominant trait. The cerebrospinal fluid of patients with HCCAA has abnormally low levels of cystatin C (Cohen *et al.*, 1983; Jensson *et al.*, 1987). Clinically, there is a selective deposition of amyloid fibrils in cerebral and spinal arteries, leading to serious brain damage and often to fatal stroke (Cohen and Jones, 1991; Grubb and Lofberg, 1985). By amino acid sequence analysis, the amyloid fibrils were found to contain a variant of cystatin C where Leucine-58 was substituted by Glutamine. A single base substitution, CAG (Gln) instead of CTG (Leu), appears to be the primary defect in this inherited disorder (Levy *et al.*, 1989).

There is compelling evidence to suggest that stefin A (a member of the family 1 cystatin superfamily) modulates the growth and metastatic potential of human angiosarcomas, malignant fibrous histiocytomas (Lah *et al.*, 1989), and poorly differentiated ovarian carcinoma (Lah *et al.*, 1992). In addition, molecular cloning of mouse epidermal cystatin A revealed that its mRNA was abundant in chemically induced skin papillomas, but reduced in carcinomas, suggesting that regulation of cystatin A expression may be important in the conversion to different types of malignancies (Hawley-Nelson *et al.*, 1988). It has also been suggested that the cysteine protease cathepsin B, and its endogenous inhibitor cystatin, play a role in metastasis (Sloane and Honn, 1984) and in tumor growth and/or progression (Sloane *et al.*, 1990). More recently, two mutations in the cystatin B gene have been found to be directly responsible for progressive myoclonus epilepsy, an autosomal recessive inherited form of epilepsy (Pennacchio *et al.*, 1996).

4. RAT CYSTATIN S

Salivary glands of mammals produce secretions important in protecting the oral cavity. The secreted cystatins are thought to play a role in this protection, constituting an important barrier against pathogens. Menaker *et al.* (1974), based on the observation that the β-adrenergic receptor agonist isoproterenol (IPR) caused enlargement of the salivary glands in rodents (Selye *et al.*, 1961), analyzed

the effect of IPR on the content of secretory proteins in whole saliva and histologic changes in rat submandibular glands. They described the presence of a conspicuous protein in the submandibular glands of rats treated with IPR, characterized by its high electrophoretic mobility in polyacrylamide gels, and called this protein **LM** (Large Mobility) **Protein** (Menaker *et al.*, 1974). The LM protein was purified and partially characterized as a 13 KDa polypeptide of unknown function, and found to be rich in aspartic acid, glutamine, and serine, but low in proline, which distinguishes it from the class of proline-rich proteins which are also induced in parotid and submandibular glands by IPR (Naito, 1981). Subsequently, a cDNA encoding the LM protein was cloned and sequenced (Shaw *et al.*, 1988). The deduced amino acid sequence revealed a high degree of sequence homology to low molecular weight cysteine protease inhibitors. Direct inhibitory assays confirmed that LM protein, isolated from saliva of IPR-treated rats, was indeed a potent inhibitor of papain and ficin but not of the bovine cathepsin B or kallikrein (a serine protease). The protein was named **rat cystatin S**, a new member of family 2 of cysteine protease inhibitors, the cystatin superfamily (Shaw *et al.*, 1988).

Cystatin S mRNA is not detected by Northern blot hybridization in the submandibular glands of 20-days old fetuses, or in the glands of rats up to three weeks of age. Between 3 and 4 weeks of age cystatin S mRNA levels increase, reaching a peak of high concentration at 28 days, and then decline to a barely

detectable level in the glands of 32 day old animals. The cystatin S gene is not normally expressed in the submandibular or parotid glands of adult rats (Shaw *et al.*, 1990). *In situ* hybridization revealed cystatin S mRNA only in the acinar cells in the submandibular glands, but not in duct cells (See a description of the structure of the submandibular gland in next section, page 15).

Administration of IPR postnatally accelerates the differentiation of acinar cells in developing submandibular glands (Ekfors *et al.*, 1972; Schneyer and Shackleford, 1963), and induces the precocious acinar cell-specific expression of the cystatin S gene (Barka *et al.*, 1986). A single injection of IPR induces cystatin S mRNA as early as 4 hours in adult animals, where the gene is not normally expressed, and high levels are detected even 72 hours later, suggesting that the half-life of the message is relatively long. Expression of cystatin S mRNA induced by IPR is detected not only in the submandibular gland but it is also detected at very low level (20-fold less) in the parotid gland. It is not detected in other tissues such as the sublingual gland, pancreas or liver (Shaw *et al.*, 1988). Induction of cystatin S mRNA by IPR in the rat submandibular gland is more pronounced in female than in male rats, and this difference in inducibility is observed also in pre-pubertal rats, suggesting that the difference is not related to the levels of circulating sex hormones (Shaw and Barka, 1989). Similarly, the expression of cystatin S mRNA analyzed by reverse transcriptase-PCR amplification (RT-PCR) and Northern blot hybrydization in the submandibular glands of adrenalectomized

and/or gonadectomized adult rats suggested that gonadal hormones are not required for IPR-induced expression of the cystatin S gene; glucocorticoids, however, may be acting in a sex-dependent manner (Chaparro *et al.*, 1994).

Cystatin S gene expression can also be induced in rats treated with the β_1-adrenergic agonist dobutamine. This induction can be blocked by the non-specific β-adrenergic antagonist propranolol, or the specific β_1-adrenergic antagonists metaprolol, protocol, and atenolol (Bedi, 1993), indicating that its expression is regulated by the β_1-adrenergic receptor pathway.

Repeated amputation of the continuously erupting lower incisor teeth causes marked enlargement of the major salivary glands (Wells, 1963), and also induces high levels of expression in the submandibular gland of a protein electrophoretically and immunologically identical to the LM protein (cystatin S) induced by IPR administration (Yagil and Barka, 1986). Teeth amputation was less effective than IPR in inducing cystatin S protein even when the gland enlargement was comparable. Neuronal mechanisms which involve sensory as well as sympathetic and parasympathetic efferent pathways seem to be responsible for the enlargement of the submandibular gland induced by the repeated amputation of the lower incisor teeth (Wells, 1963), and probably for the increased expression of cystatin S.

It has been reported recently that papain (a cysteine protease) administered orally to adult rats induces submandibular and parotid gland enlargement,

synthesis of cystatin S in the submandibular gland, and its secretion in saliva. The induction of cystatin S by papain, and the enlargement of the salivary glands was inhibited by metroprolol (a selective β_1-receptor antagonist) (Naito *et al.*, 1992), and it was suggested that this biological response is a first line of defense which protects the oral cavity from injury caused by exogenous proteases. These results also suggest that chemosensory inputs might participate in the regulation of cystatin S gene expression in the submandibular gland (Naito *et al.*, 1992). The analysis of the adrenergic mechanisms involved in rat submandibular gland hypertrophy induced by lower incisor amputation, intraoral administration of papain and IPR treatment showed that guanethidine (an adrenergic neuronal blocking agent), significantly reduced the salivary gland enlargement induced by papain or teeth amputation but not by IPR treatment. Propanolol antagonized the effects of IPR and papain but not teeth amputation (Brenner and Stanton, 1970). These results suggest that neuronal mechanisms involved in the hypertrophy of rat submandibular glands and probably in cystatin S gene expression induced by IPR, intraoral papain administration or lower incisor teeth amputation are not identical although they may share common elements.

In summary, rat cystatin S gene, a member of the family 2 of the cysteine protease inhibitors, the "Cystatin Superfamily", has a very defined pattern of expression during development. Its expression is tissue-specific and cell type-specific, and can be induced by β_1-adrenergic agonists in adult animals where it is

not normally expressed. Cystatin S has been characterized biochemically and enzymatically, but its physiological role and how its expression is regulated are still not completely understood.

Since the effects of the β-adrenergic agonist isoproterenol (IPR) in the rat submandibular glands are not restricted to cystatin S gene expression, and it has other profound effects on the postnatal differentiation of the parotid and submandibular glands, the next sections will review some of these effects, the autonomic nervous system innervation and the pattern of normal development of the rat submandibular gland.

5. POSTNATAL DEVELOPMENT OF THE RAT SUBMANDIBULAR GLAND

The major salivary glands of mammals, the parotid, submandibular and sublingual glands, play an important role in maintaining oral health. Mammalian salivary glands share a common developmental pattern, including a delayed initiation of development and differentiation (reviewed in Denny *et al.*, 1997).

The rat submandibular gland undergoes a complex process of growth and differentiation during the postnatal period, regulated by the interplay of hormones and the autonomic nervous system. It reaches full maturity by three months of age. The adult salivary gland may be considered as consisting of four major components: (1) acini, (2) intercalated ducts, (3) granular convoluted tubules

(GCT), and (4) striated duct-excretory ducts. The compartments differ not only in function, but also in response to regulatory inputs (reviewed in Barka, 1990).

The main function of the *acinar cells* is the production of primary saliva, which is isotonic with the plasma. The major regulation of acinar cell function is provided by the autonomic nervous system (Emmelin, 1964). Some hormones, notably estrogens, androgens and thyroid hormones also affect the function of acinar cell (reviewed in Gresik, 1980) The *intercalated duct cells* may serve as stem cells for both acinar cells and GCT cells during normal and hyperplastic growth. Secretory granules of unknown composition occur in some intercalated duct cells. The *granular convoluted tubule* (GCT) compartment, particularly in the mouse, synthesizes and secretes a number of biologically active polypeptides (Barka, 1980). Secretion in the GCT cells is mediated by α-adrenergic receptors (Abe and Dawes, 1978; Anderson *et al.*, 1995).The major function of the cells in the *striated-secretory duct* is the regulation of ion composition and water content of saliva. Striated duct cells have limited secretory activity but contribute to the very high level of the serine protease kallikrein in saliva (Barka, 1990).

The postnatal development of the rat submandibular gland may be divided into two phases (Jacoby and Leeson, 1959): During the first phase, from birth to approximately 6 weeks of age, the secretory units of adult type, *the acini*, are formed from rudimentary secretory units known as "terminal tubules". During the second phase, between 6 and 12 weeks of age, a segment of the intralobular duct

develops into *the granular convoluted tubule*, and the gland attains its adult characteristics.

Postnatal differentiation of acini in the submandibular gland of 2 to 42 day old rats was analyzed by Chang in 1973, and the following interrelationships of cells during the formation of the acinus from terminal tubules were postulated: At birth the terminal tubules consist mainly of terminal tubule cells and proacinar cells. Acini develop from the terminal tubules as cell buds consisting mostly of proacinar cells at two days of age. During the first week, following mitosis the proacinar cells give rise to acinar cells, and thus at the end of the first week the cell buds are comprised predominantly of acinar cells. After depletion of the proacinar cell population between the first and second week, the terminal tubule cells become the immediate precursor of acinar cells. By six weeks, when acini formation is complete, the terminal tubule cells disappear. The acinar cells which originate from either the proacinar cells or the terminal tubule cells divide repeatedly. However, the proliferative activity of acinar cells decreases rapidly after seven days, during early postnatal development (Chang, 1973).

6. THE AUTONOMIC NERVOUS SYSTEM AND AUTONOMIC INNERVATION OF THE SUBMANDIBULAR GLAND

The *Autonomic Nervous System* (ANS) supplies innervation to the internal organs (smooth muscles, cardiac muscle, glands, etc.). The ANS has two main divisions: the *sympathetic* and the *parasympathetic* nervous systems. Sympathetic ganglia are arranged in a cord along the vertebral column and receive their neuronal input from cells located in the intermediolateral column of the thoracolumbar portions of the spinal cord. Parasympathetic ganglia, in contrast, are located very close to the organs they innervate and receive their input from fibers arising from cells in nuclei of the brainstem and sacral spinal cord. According to their positions relative to the ganglia, fibers innervating a ganglion are called *preganglionic* and those arising from it are *postganglionic* (see **Figures 1-1 & 1-2**).

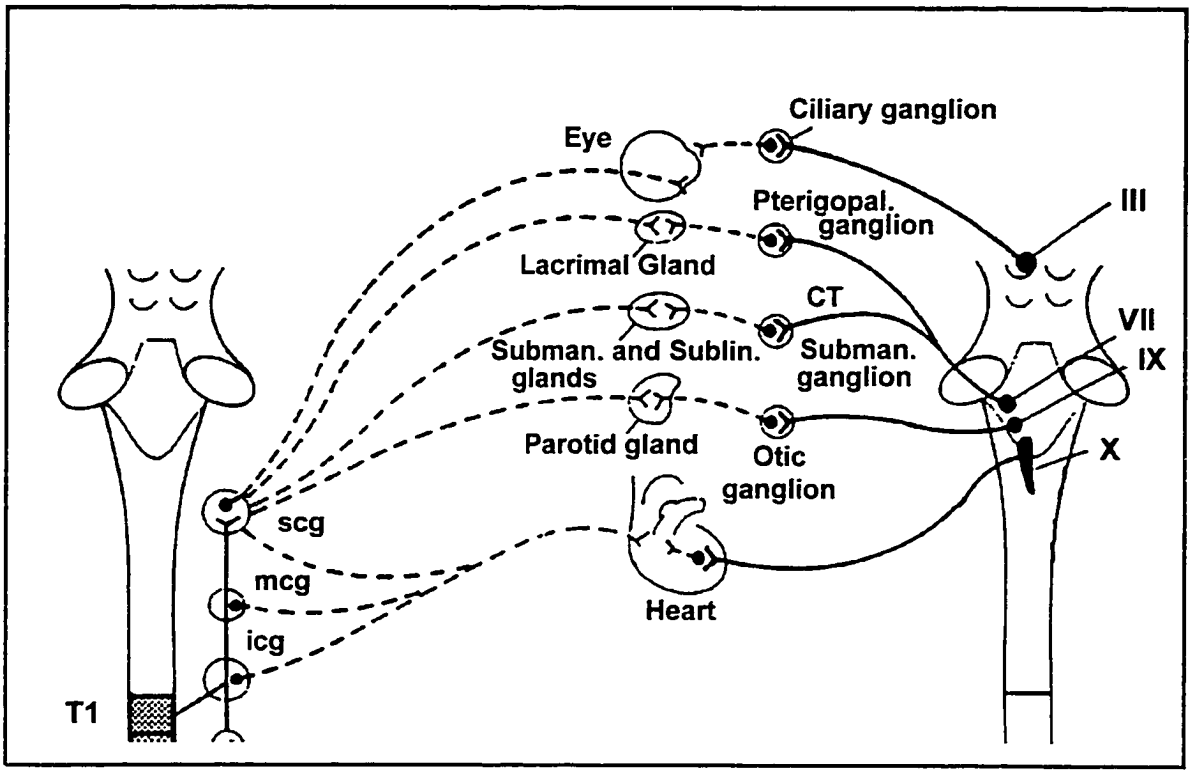


Figure 1-1. Partial representation of the organization of the autonomic nervous system. The sympathetic system is shown on the left and the parasympathetic system on the right. Abbreviations: **T1**, first segment of the thoracic spinal chord; **scg**, superior cervical ganglion; **mcg**, middle cervical ganglion; **icg**, inferior cervical ganglion; **III-IX**, cranial nerves; **CT**, chorda tympani nerve. (Modified from Shepherd, 1988).

In both branches of the ANS the chemical transmitter for the synapse between the preganglionic and the postganglionic fibers is *acetylcholine*. The transmitter between the parasympathetic postganglionic fibers and the effector organs is also acetylcholine, but the transmitter at the synapses between the sympathetic postganglionic fibers and the effector organs is *norepinephrine* (see **Figure 1-2**).

In addition to norepinephrine and acetylcholine, the autonomic nerve terminals release several neuropeptides that play an important role in the regulation of submandibular gland metabolism. The distribution of some of these neuropeptides in the sympathetic and parasympathetic nerve terminals innervating the rat submandibular gland is listed in **Table 1.** The function of neuropeptides potentially relevant to the regulation of cystatin S gene expression will be discussed in Chapter III.

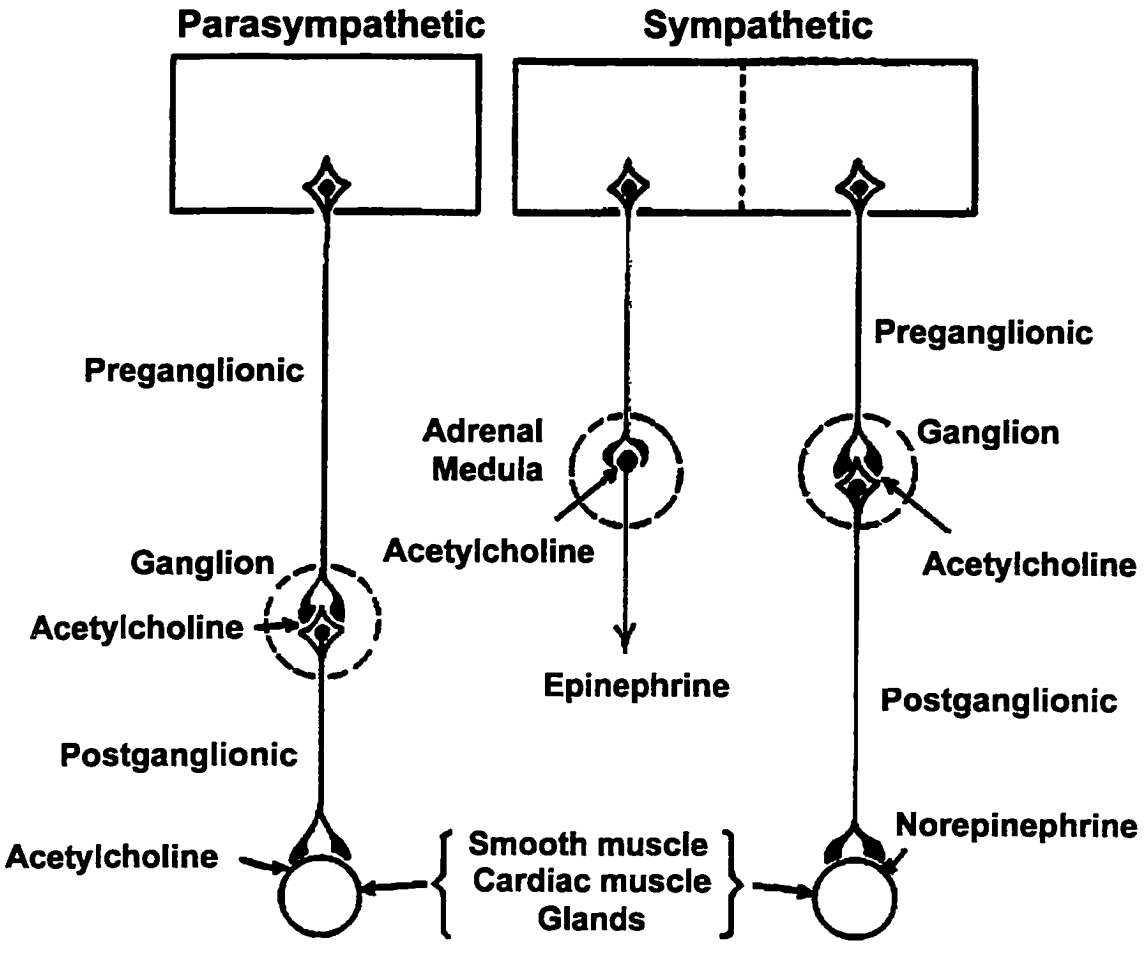


Figure 1-2. Chemical transmitters in the autonomic nervous system. (Modified from Pansky *et al.*, 1988).

Table 1. Neuropeptide phenotype in autonomic nerve terminals innervating the rat submandibular gland.

Sympathetic Neuropeptides	References
Neuropeptide Y (NPY)	1,2,3, 4
Vasoactive intestinal peptide (VIP)	2, 5, 6, 7
Galanin	5
Calcitonin gene-related peptide (CGRP)	8
Somatostatin	6, 7
Enkephaline	9
Parasympathetic Neuropeptides	
Neuroeptide Y (NPY)	1, 4
Vasoactive intestinal peptide (VIP)	12, 13, 16
Substance P (SP)	10, 11, 13
Neurokinin A	12
Galanin	15
Calcitonin gene-related peptide (CGRP)	16
Pituitary adenylate cyclase activating peptide (PACAP)	17

1. (Ekström *et al.*, 1996). **2.** (Tyrrel and Landis, 1994). **3.** (Lundberg *et al.*, 1982b). **4.** (Schultz, 1994). **5.** (Hyatt-Sachs *et al.*, 1996). **6.** (Lundberg *et al.*, 1982a). **7.** (Hokfelt *et al.*, 1977). **8.** (Salo *et al.*, 1995). **9.** (Kondo *et al.*, 1988). **10.** (Ekström *et al.*, 1989). **11.** (Ekström, 1987). **12.** (Virta *et al.*, 1992). **13.** (Ekström *et al.*, 1984). **14.** (Lundberg *et al.*, 1980). **15.** (Kanopka *et al.*, 1992). **16.** (Ekström *et al.*, 1988). **17.** (Mirfendereski *et al.*, 1997).

Norepinephrine activates multiple distinct signaling pathways in salivary gland cells including: β-adrenergic receptor-mediated stimulation of adenylate cyclase, α_2-adrenergic receptor-mediated inhibition of adenylate cyclase, and α_1-adrenergic receptor-mediated stimulation of phospholipase C (Bylund *et al.*, 1982).

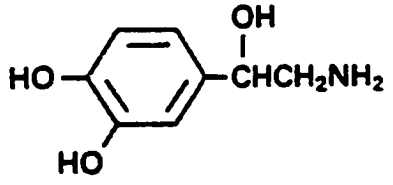
Both branches of the ANS innervate the major salivary glands and constitute a primary factor in controlling salivary gland functions. The *superior cervical ganglion* receives preganglionic fibers arising from the first segment of the thoracic spinal cord (T1), and its postganglionic fibers innervate the parotid, submandibular and sublingual glands (**Figure 1-1**). The submandibular gland is also innervated by the parasympathetic nervous system by postsynaptic fibers from the submandibular ganglion. Parasympathetic preganglionic fibers from cranial nerve VII innervate the submandibular ganglion, via the *chorda tympani* nerve (**Figure 1-1**).

7. EFFECTS OF THE β-ADRENERGIC AGONIST ISOPROTERENOL (IPR) ON THE RAT SUBMANDIBULAR GLAND

The autonomic nervous system has a profound effect on the postnatal differentiation and growth of acinar cells in the parotid and submandibular glands of mice and rats. This regulation is mediated primarily by β_1-adrenergic receptors

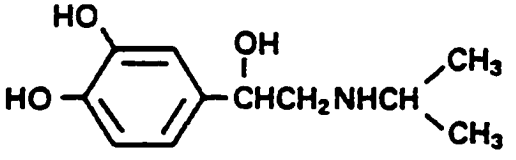
and can be reproduced experimentally by the β-adrenergic agonist **isoproterenol (IPR)**. In contrast to other tissues such as lung and heart, which contain both β_1- and β_2-adrenergic receptors, the submandibular gland appears to contain only β_1-adrenergic receptors which bind epinephrine and norepinephrine with approximately equal affinities, but their affinity for IPR is about 10 times higher (Bylund *et al.*, 1981).

A



Norepinephrine

B



Isoproterenol

Figure 1-3. Chemical structure of Norepinephrine and the β-adrenergic agonist isoproterenol.

IPR profoundly affects the growth and differentiation of the submandibular and parotid glands of mice and rats (Barka, 1965a; Brenner and Wulf, 1981; Brown-Grant, 1961; Selye *et al.*, 1961); it stimulates DNA and RNA synthesis (Barka, 1965b; Radley, 1968; Sheetz and Menaker, 1979), produces changes in the structure of secretory granules (Segawa *et al.*, 1991), and induces the secretion of specific sets of proteins (Bedi, 1991b; Humphreys-Beher, 1984; Mehansho and Carlson, 1983; Naito, 1981). IPR has also been shown to have a profound effect on gene expression in salivary glands, altering the expression of protooncogenes (Barka *et al.*, 1986; Kousvelari *et al.*, 1988) and several secretory genes like proline-rich proteins (PRP) (Ann *et al.*, 1987), glutamine/glutamic acid rich proteins (GRP) (Matsuura and Hand, 1991) and the family 2 protease inhibitor, **cystatin S** (Shaw and Barka, 1989; Shaw *et al.*, 1990).

The rate of cell replication in the parotid and submandibular glands of adult rats and mice is very low. Most of the acinar cells in the gland are considered to be in the quiescent G_0 phase. A single injection of IPR stimulates DNA synthesis and cell division of the acinar cells in these glands with a lag period of 24-28 hours (Barka, 1965a; Barka, 1965b). When animals are treated chronically with IPR, stimulation of DNA synthesis declines, but a marked and reversible hyperplastic and hypertrophic enlargement of the glands is produced (Barka, 1965a; Brown-Grant, 1961). During postnatal development, the degree of stimulation of DNA synthesis is inversely proportional to the rate of cell replication in unstimulated

glands, and IPR does not stimulate DNA synthesis in the submandibular glands of 1-5 day old animals. However, during this developmental period, treatment with the drug for several days causes an apparent acceleration of acinar cell differentiation (Ekfors *et al.*, 1972; Schneyer and Shackleford, 1963; Yamashina and Barka, 1972). All these effects are mediated by the adenylate cyclase-cAMP system and calcium ions. Administration of IPR leads to a rapid increase in cAMP (Humphreys-Beher and Schneyer, 1986; Rajakumar and Scarpace, 1994), and intracellular calcium in rat salivary glands (Doughney *et al.*, 1987; Mills *et al.*, 1993; Quissell *et al.*, 1993). IPR also activates cAMP-dependent protein kinase A (PKA type I or II) in rat submandibular and parotid glands (Quissell *et al.*, 1993). PKA activated by cAMP phosphorylates a variety of cytosolic and nuclear proteins leading to cellular exocytosis, specific protein phosphorylation, and the transcriptional activation of specific sets of genes (Kobilka, 1992; Lalli and Sassone-Corsi, 1994). (See **Figure 1-4**).

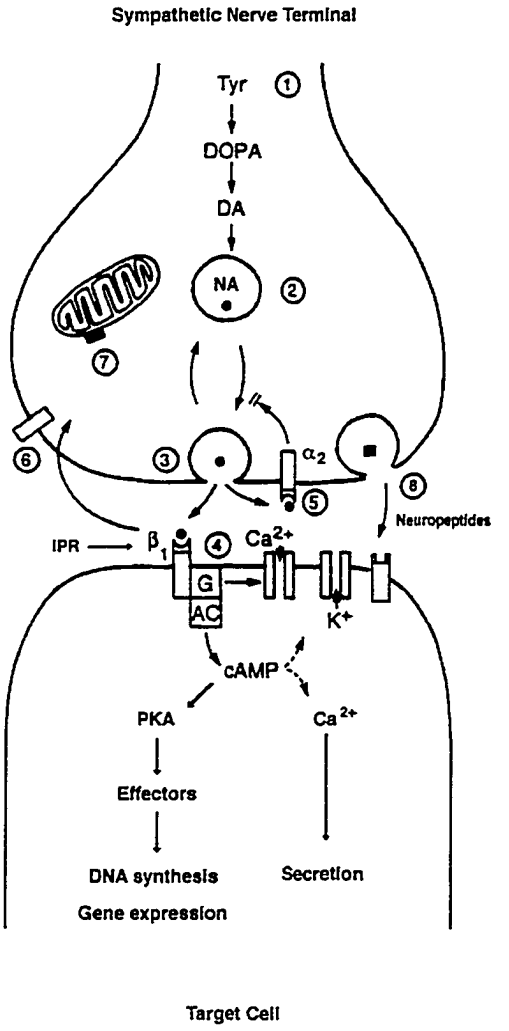


Figure 1-4. Molecular mechanisms at the β-adrenergic synapse. 1-2. Synthetic pathway from tyrosine (Tyr) to norepinephrine (NE). **3.** Release by exocytosis. **4.** Binding of the agonist (NE, IPR) to β_1-adrenergic receptors, increase in intracellular cAMP and Ca^{++}, leading to exocytosis, DNA synthesis and expression of specific sets of genes. **5.** Binding of NE to presynaptic α_2-adrenergic receptors and inhibition of NE release. **6.** Reuptake, which terminates NE action. **7.** Degradation of NE by monoamin oxidase (MAO). **8.** Co-release of neuropeptides and binding to specific receptors (Adapted from Shepherd, 1988).

8. OVERVIEW AND AIMS OF THE PROJECT

The complex pattern of regulation of the rat cystatin S gene suggest a very important function of the protein. The cystatin S protein has been shown to be a potent inhibitor of the cysteine proteases papain and ficin but not of bovine spleen cathepsin B, another cysteine protease, or kallikrein, the major serine protease in rat submandibular glands (Barka *et al.*, 1988). Since cystatin S is secreted in saliva it has been assumed that it has a protective role in minimizing the potentially harmful effects of proteinases of bacterial or cellular origin in the oral cavity (Barka, 1990; Shaw *et al.*, 1988) . However, neither the function of cystatin S protein nor its physiological targets are known. It is also unknown if cystatin S has any intracellular activity inhibiting lysosomal proteases and contributing to cell proliferation and/or cell growth in the submandibular gland (Barka, 1990). Detailed information about its highly regulated expression during submandibular gland development and the precise mechanism of this regulation by neuronal input is not yet available.

The original goal of this project was to elucidate the molecular mechanisms responsible for the regulation of the rat cystatin S gene in the submandibular gland by characterization of cis-acting elements in the 5'-flanking region of the gene mediating its expression. The experiments were designed to provide information about the response elements in the 5'-flanking region of the cystatin S gene that mediate its transcriptional activation.

The establishment of an *in vitro* system represented a key element in experiments designed to compare cystatin S gene expression in response to different external stimuli and to test the functionality of potential regulatory sequences by their ability to regulate expression of a reporter gene in stably or transiently transfected cells. Cystatin S gene expression *in vitro* in primary cultures of submandibular gland cells was analyzed by Western blot analysis and reverse transcriptase-PCR amplification (RT-PCR) of the cystatin S mRNA (see Chapter II for a detailed description of the methodology). Results of those experiments demonstrated that cystatin S gene expression is not induced *in vitro* by IPR, dibutyryl-cAMP, forskolin, β-estradiol or dexamethasone. The results in **Figure 1-5** show that cystatin S protein was not detected in the submandibular glands of untreated adult female rats (**lane 1**). However, high level of expression of the protein was induced 24 hours after a single injection of IPR (**lane 2**). When the animals were treated *in vivo* with IPR, the submandibular glands removed 24 hours later and then, primary cultures of submandibular gland cells incubated for approximately 22 hours, cystatin S protein was still detected, suggesting that the acinar cells remained functional after this incubation period (**lane 4**). No expression of cystatin S was detected in primary cultures of submandibular gland cells from untreated animals indicating that the manipulation of the cells during the primary culture process had no effect on cystatin S gene expression (**lane 3**). Cystatin S protein was not detected in control non IPR-treated primary culture of

submandibular gland cells (**lane 5**) or in primary culture cells treated *in vitro* with IPR (**lane 6**).

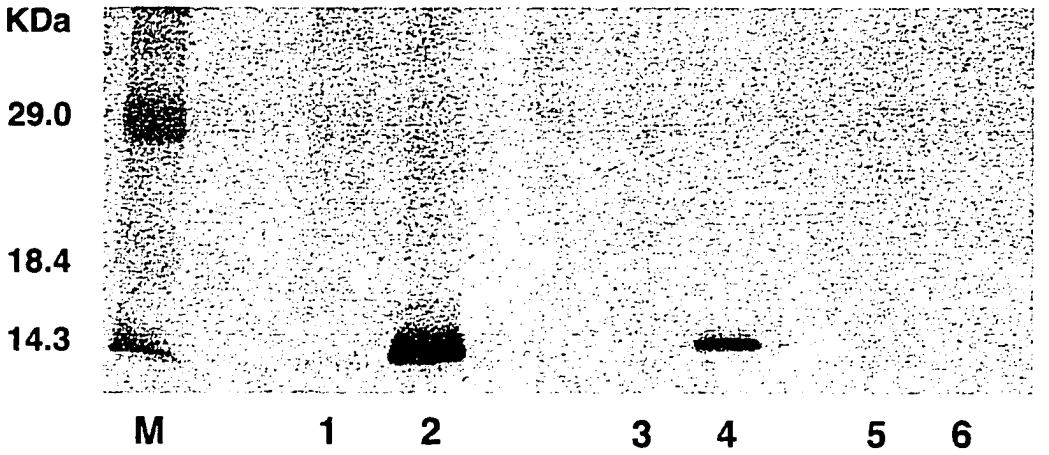


Figure 1-5. SDS-PAGE and immunoblots of proteins from rat submandibular gland primary cultures treated or untreated with IPR. Submandibular gland cells were dispersed enzymatically, cultured and treated for 22 h as described in General Methods. Proteins extracted from the submandibular gland cells (20 μg on lanes 1-2; and 5 μg on lanes 3-6) were electrophoresed on 12 % polyacrylamide gels, electrotransferred onto nitrocellulose paper and immunostained using anti-LM antiserum. **M.** Prestained molecular weight markers. **Lane 1.** Whole gland extract from a control, untreated adult female rat. **Lane 2.** Whole gland extract from an IPR-treated adult female rat. **Lane 3.** Primary culture of cells from a control, untreated female submandibular gland. **Lane 4.** Submandibular gland cells from a female rat treated with IPR *in vivo*, 24 h before dispersing and culturing the cells with no additional treatment *in vitro.* **Lane 5.** Control, untreated primary culture of submandibular gland cells. **Lane 6.** Primary culture of submandibular gland cells treated *in vitro* with IPR (10^{-4} M).

The results also demonstrated that cystatin S gene expression was not induced *in vitro* by IPR, dibutyryl-cAMP, forskolin, β-estradiol or dexamethasone (**Figure 1-6**)

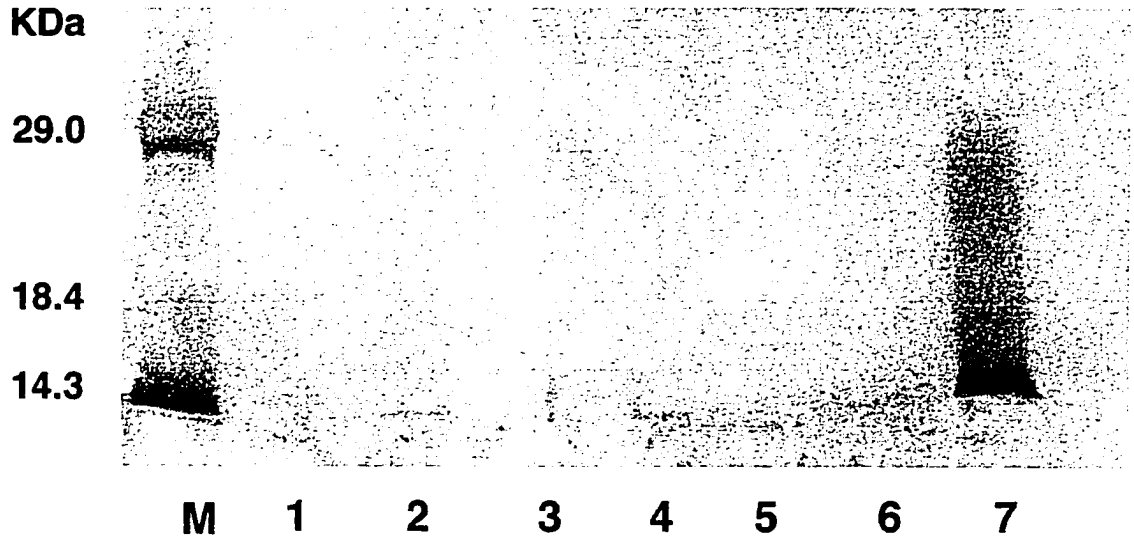


Figure 1-6. SDS-PAGE and immunoblots of proteins from submandibular gland primary cultures treated with several potential inducers of cystatin S gene expression. Submandibular gland cells were dispersed enzymatically and primary culture established and treated for 22 h as described in General Methods. Proteins extracted from the submandibular gland cells (7.5 μg) were electrophoresed on 12 % polyacrylamide gels, electrotransferred onto nitrocellulose paper and immunostained using anti-LM antiserum. **M.** Prestained molecular weight markers. **Lane 1.** Control, untreated primary culture of submandibular gland cells. **Lanes 2-6.** Primary culture of submandibular gland cells treated *in vitro* with: **Lane 2,** IPR (10^{-4} M). **Lane 3,** dibutyryl-cAMP (10^{-3} M). **Lane 4,** forskolin (10^{-4} M). **Lane 5,** β-estradiol (10^{-8} M). **Lane 6,** dexamethasone (10^{-6} M). **Lane 7.** Positive control, whole gland extract from an IPR-treated adult female rat.

Cystatin S gene expression induced by IPR *in vitro* in primary cultures of submandibular gland cells was also analyzed by RT-PCR amplification of the cystatin S mRNA. The results in **Figure 1-7** confirmed the observations from the western blot analyses. In contrast with the high level of expression of the cystatin S gene in the submandibular glands of female rats treated with IPR *in vivo* (**positive control, lane 3**), there were no differences in the levels of cystatin S mRNA between untreated cells (**lanes 4-6**) and cells treated *in vitro* with IPR (**lanes 7-9**).

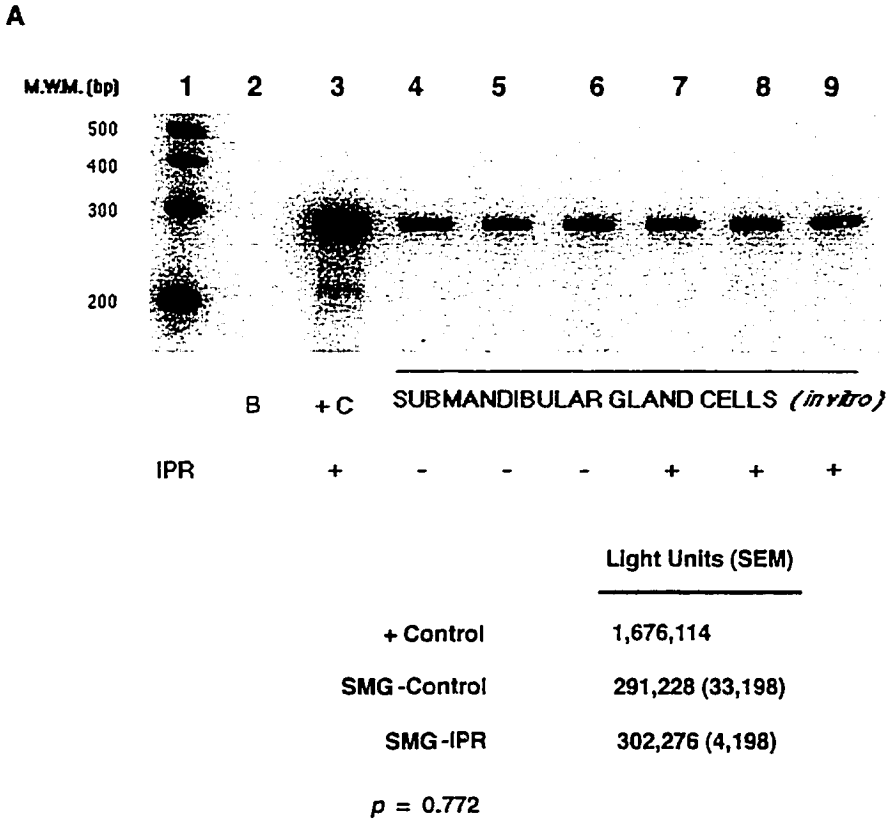


Figure 1-7. RT-PCR analysis of cystatin S gene expression *in vitro* in primary cultures of rat submandibular gland cells treated with IPR. Total RNA was used as a template in the RT-PCR reaction, and the products labeled, and electrophoresed as described in General Methods. **Lane 1**, molecular weight markers. **Lane 2**, Blank (no template RNA). **Lane 3**, a positive control (100 ng of total RNA from the submandibular gland of an adult female rat treated with IPR). **Lanes 4-6**, total RNA (250 ng) from primary cultures of untreated submandibular gland cells. **Lanes 7-9**, total RNA (250 ng) from primary cultures of submandibular gland cells treated with IPR (10^{-5} M). The RT-PCR product s were quantitated in the PhosphorImager and a Student's *t*-test for independent samples showed that the differences between primary culture control and IPR-treated cells cystatin S gene expression are not significant.

Stable and transient transfection of the rat submandibular cell lines 2C2 and A5 demonstrated that fragments of the 5'-flanking region of the cystatin S gene (up to 2 Kb) failed to show regulated expression of the luciferase-reporter gene, in response to IPR (data not shown).

Taken together these results suggested that *in vivo* in addition to the stimulation of β_1-adrenergic receptors the submandibular gland requires additional factor(s) for cystatin S gene expression. Obviously, one of the circuits that is interrupted when the submandibular gland is removed from the animal is its communication with the autonomic nervous system. Is it possible that factors from the autonomic nervous system (neurotransmitters/neuropeptides) participate in the cystatin S gene expression mediated by stimulation of β_1-adrenergic receptors?

The lack of response of the cystatin S gene to IPR treatment in primary cultures of rat submandibular gland cells, and previous reports suggesting that neuronal mechanisms which probably involve sensory as well as sympathetic and parasympathetic efferent pathways seem to be involved in the increased expression of cystatin S in the rat submandibular gland (Naito *et al.*, 1992; Yagil and Barka, 1986) led to the formulation of the following hypothesis: *the autonomic nervous system (sympathetic and parasympathetic) participates in the regulation of the cystatin S gene expression induced by the β-adrenergic agonist isoproterenol in the rat submandibular gland.*

This hypothesis can be tested experimentally by examining the effect of surgical and chemical sympathectomy , and surgical parasympathectomy on the IPR-induced expression of the cystatin S gene in the submandibular glands of female adult rats. Since the sympathetic innervation is not fully developed in newborn rats (Bottaro and Cutler, 1984; Cutler *et al.*, 1981), the submandibular gland offers an excellent experimental model for the analysis of the correlation between cystatin S gene expression induced by IPR and sympathetic innervation during early development of the submandibular gland.

The specific aims of the study are:

1. To analyze the effect of bilateral or unilateral sympathectomy on the expression of the cystatin S gene induced by isoproterenol in the submandibular glands of adult rats.

2. To analyze the effect of "chemical sympathectomy", produced by treatment with reserpine, on the expression of the cystatin S gene induced by isoproterenol in adult rat submandibular glands.

3. To analyze the expression of the cystatin S gene induced by isoproterenol during early postnatal development of the rat submandibular gland before and

after the sympathetic nerve fibers from the superior cervical ganglion innervate the gland.

4. To investigate the effect of sympathectomy on the expression of the cystatin S gene induced by isoproterenol during early postnatal development of the rat submandibular gland.

5. To investigate the effect of surgical parasympathectomy of adult rat submandibular glands, by sectioning the chorda tympani / lingual nerve, on the expression of the cystatin S gene induced by isoproterenol.

6. To analyze the effect of glossopharyngeal denervation on the expression of the cystatin S gene induced by isoproterenol in the submandibular gland of adult rats.

CHAPTER II

GENERAL METHODS

Materials

T7 RNA polymerase, RNase-free DNase I, and restriction enzymes were from New England Biolabs; [^{32}P] UTP (3000 Ci/mmole) was obtained from New England Nuclear (NEN). DL-isoproterenol-HCl [(3,4-dihydroxy-alpha-(isopropyl-amino) methyl] benzyl alcohol, forskolin, estradiol benzoate, cycloheximide and Tri-Reagent for RNA extraction were purchased from Sigma (St Louis, MO). Dexamethasone sodium phosphate was purchased from American Reagents Lab Inc (Shirley, NY). All other materials were purchased from commercial sources and were of the highest purity available.

Animals

Adult female Sprangue-Dawley rats (body weight 150-200 g) purchased from Charles River Laboratories (Wimintong, MA) were used in these studies. Since IPR induction of cystatin S mRNA in adult submandibular glands is more pronounced in female than in male rats (Shaw *et al.*, 1990), only female rats were used in this study. The animals were kept in a temperature and humidity controlled

environment (12 h light, 12 h dark cycle), and had free access to water and standard laboratory chow at Mount Sinai's Center for Lab Animal Sciences (CLAS). All experimental protocols were reviewed by the Mount Sinai Institutional Animal Care and Use Committee and conducted in accordance with the NIH guidelines for the care and use of laboratory animals (NIH Pub. No. 85-23, revised 1985).

SURGICAL PROCEDURES

Surgical denervation procedures were performed by Dr. Wan-Hua Amy Yu from the Department of Cell Biology and Anatomical Sciences of the City University of New York Medical School New York, N.Y.

1. Removal of superior cervical ganglion

1) In adult female rats surgical sympathectomy was performed using ketamine (50 mg/kg, *i.p.)* and xylazine (5 mg/kg, *i.m.*) anesthesia. A midline incision was made through the skin at the anterior cervical region. The cervical sympathetic nerve trunk was exposed and dissected free from the carotid sheath up to the bifurcation of the carotid artery. After localizing the superior cervical ganglion (SCG), the entire ganglion was cut away from the sympathetic trunk with a pair of scissors. In the bilateral sympathectomy experiments, the superior cervical ganglion was removed from both sides; in the unilateral experiments, the right superior cervical ganglion was excised, and the left ganglion was left intact

and unperturbed. The skin incision was then closed with stainless steel wound clips. Sham-operated animals were treated similarly except that the SCG was not excised.

2) Surgical unilateral sympathectomy was also performed on one-day old rats (day of birth is day 0) anesthetized by hypothermia. A midline incision was made through the skin at the anterior cervical region. The right superior cervical ganglion at the bifurcation of the common carotid artery was identified under a dissecting microscope and crushed with a pair of Dumont # 5 forceps. The skin incision was closed with 7-0 silk suture.

2. Chorda tympani and Glossopharyngeal Denervations

Surgical parasympathectomy of adult female rats was performed using ketamine (50 mg/kg, *i.p.)* and xylazine (5 mg/kg, *i.m.*) anesthesia. Because of the difficulty of removing the submandibular ganglion completely in rats, we chose to sever the chorda tympani/lingual (CT/LN). A midline incision was made through the skin at the anterior cervical region. In the bilateral parasympathectomy experiments, CT/LN was severed on both sides; in the unilateral experiments the right CT/LN were severed, and the left nerves were left intact and unperturbed as an internal control. Unilateral glossopharyngeal denervation was made by severing the nerve on the right side; the left side nerves were left intact and unperturbed.

The skin incision was then closed with stainless steel wound clips. Sham-operated animals were treated similarly except that the nerves were not severed.

Isoproterenol treatment

Bilaterally sympathectomized and bilaterally parasympathectomized adult female rats were given one intraperitoneal injection of 0.15 μmol/g body weight of IPR on the third or the fourteenth day after surgery. IPR was dissolved in 0.1% (w/v) sodium metabisulfite in 0.85% (w/v) NaCl. Sham-operated animals were injected with 0.85% NaCl alone or with IPR as above. Unilaterally denervated adult rats were injected only on the fourteenth day after surgery. The animals were killed under excess of CO_2 24 h after IPR injection, the submandibular glands were removed, weighed, frozen in liquid nitrogen and kept at -70° C until the RNA was extracted; before freezing, a portion of the gland was fixed in 4% paraformaldehyde for morphological studies.

Female pups unilaterally sympathectomized at one day of age, were treated with a single *i.p* injection of IPR, as described above, at 4 and 8 days of age, and 24 hr later the animals were anesthetized by hypothermia, the submandibular glands were removed, frozen in liquid nitrogen, and kept at -70° C until RNA was extracted.

Reserpine treatment

"Chemical sympathectomy" was performed by treating adult female with reserpine. Rats were given two daily injections of 0.5 mg/kg body weight of reserpine (Bylund *et al.*, 1981). Then, 24 hours after the second reserpine injection, a single dose of IPR was given at the concentration noted above. Twenty four hours later, the animals were killed and the submandibular glands were removed for RNA extraction and morphological studies.

Cycloheximide Treatment

Two animals per group were given a single subcutaneous injection of cycloheximide (CHX, 5 mg/kg body wt) (Murayama *et al.*, 1991; Sousa Neto *et al.*, 1990), and/or IPR as described above. The submandibular glands were removed 6 hr after the injection, and frozen in liquid nitrogen and kept at -70° C until RNA was extracted.

Microscopy

Submandibular glands were removed, weighed, and samples of the glands were processed for microscopic examination. Four micron thick sections stained with hematoxylin and eosin (H & E) and 3 micron thick sections stained by Periodic Acid Shiff (PAS) were prepared by personnel from the Mount Sinai's Medical Center Department of Pathology from tissues fixed in 4% paraformaldehyde, and embedded in paraffin.

RNA isolation and preparation of riboprobes

Total RNA was extracted from submandibular glands using a Tri-Reagent kit (Sigma), according to a modified procedure of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). The integrity of the RNA was monitored by electrophoresis in 1% agarose gels containing ethidium bromide; RNA was quantitated by measuring the absorbance at 260 nm using a Beckman DU 640 Spectrophotometer, assuming absorbance of one OD_{260} unit equal to 40 μg/ml RNA. The ^{32}P- labeled cystatin S riboprobe was prepared by an *in vitro* transcription reaction using as a template a PCR product which contains the T7 promoter. This template was generated from a plasmid containing the cystatin S cDNA (clone 1a) (Shaw *et al.*, 1988), and the T7 promoter sequence appended at the 5'-end of the downstream primer. The upstream primer, Fcys S-1 (5'-CTAGGTCACTTTCTGGGTGGC-3') primes at position +52 to +72 of the cystatin S cDNA. The downstream primer, T7CysS-1 (5'-AGAATTCTAATACGA

CTCACTATA**G**GGAGGTATGAGAGCTGGAGCTCAGC -3') contains the T7 promoter sequence (underlined) with the transcription start site in bold; it primes at position +372 to +392 in the cystatin S cDNA sequence. A similar strategy was used to generate a riboprobe for cyclophilin from the plasmid p1B15 that contains the rat cyclophilin cDNA sequence (Danielson *et al.*, 1988), amplifying a fragment corresponding to position +300 to +420 in the cDNA sequence.

Northern blot analyses

Total RNA, extracted from submandibular glands was electrophoresed in 5% polyacrylamide-urea denaturing gels (Stoeckle and Guan, 1993). The specified amount of total RNA, dissolved in 0.1 mM ethylenediamine-tetraacetic acid (EDTA), pH 8.0, was electrophoresed in 5% polyacrylamide-urea denaturing gels, in 1X TBE (89 mM Tris-borate and 2 mM EDTA, pH 8.0), using a minigel system from Hoefer Scientific Instruments for 1 hr at 250 Volt., and electrotransferred in 0.5X TBE for 1 hr at 250 mamps onto Gene Screen Plus membranes. This time was predetermined to be sufficient for complete transfer of the 900 nucleotide (nt) long cystatin S mRNA. Molecular weight markers were 5000 c.p.m. of a ^{32}P-labeled DNA ladder. RNA blots were prehybridized, hybridized overnight at 65°C with the ^{32}P-labeled cystatin S riboprobe, and washed according to Shaw *et al.* (Shaw *et al.*, 1988).

Duplicate filters were hybridized to a ^{32}P-labeled probe specific for rat cyclophilin RNA, a constitutively expressed RNA (Jakubowski *et al.*, 1991). Cyclophilin has been used as an internal standard to quantitate the specificity of changes in calcitonin and calcitonin gene-related peptide mRNA levels in the CA-77 cell line (Collignon *et al.*, 1992), as an internal standard in RT-PCR studies measuring the mRNAs encoding 5-HT1A and 5-HT2A proteins in human hippocampus (Burnet *et al.*, 1994), and as a standard for normalization in

experiments examining the effects of steroid hormones and β-adrenoreceptor agonists on the regulation of the rat cystatin S gene (Chaparro *et al.*, 1994).

Quantitative analyses of the Northern blots

Quantitative analyses of Northern blots were performed using the PhosphorImager (Molecular Dynamics). Filters on the phosphor screen were exposed overnight and after scanning the image, the Grey/Color Adjust window was used to improve the visibility of the bands. The bands to be quantitated were identified by drawing a rectangle around each band. The rectangle was drawn around the first band to be quantitated and duplicated around the other bands in order to have the same volume for all the samples to be compared. The Volume Quantitation Mode was used and the background corrected automatically by using Local Average, which uses the average pixel value found in the outline of each rectangle as the background value. In all experiments, samples to be compared were hybridized, exposed to the phorphor screen and quantitated simultaneously. The raw data from all the experiments are tabulated in Appendix 2.

Standard Curve for Range of signal Detection

In order to confirm that the signal in the PhosphorImager was in the linear range of detection for the amount of RNA used in the quantitative analyses of cystatin S gene expression by Northern blots (1 μg of total RNA/ lane), a standard

curve was generated by dot blot analyses of increasing concentrations of total RNA extracted from the submandibular glands of IPR-treated adult female rats. Total RNA (0, 0.25, 0.5, 1.0 and 1.5 μg) was denatured and spotted directly on to Gene Screen Plus membranes and hybridized to the ^{32}P-labeled cystatin S riboprobe as described above. The signal was quantitated in the PhosphorImager as described previously and the data plotted as μg of total RNA *vs* Intensity of the signal (Light Units).

Statistical analyses

The significance of the differences in cystatin S gene expression in the submandibular glands between sham operated and bilaterally sympathectomized rats, control and reserpine-treated animals, and sham operated and bilateral parasympathectomized rats was calculated by the Student's *t*-test for independent samples using the SPSS 6.1.2S computer program. Data from unilateral denervation experiments (unilateral sympathectomy of adult and 4 and 8 day-old animals, unilateral parasympathectomy and unilateral glossopharyngeal denervations) were analyzed by the Student's *t*-test for paired samples. The type I error level was set at $p<0.05$ for each statistical test. The significance of the differences in the IPR-induced expression of cystatin S gene during early development (3, 4, 5, 6 and 8 days of age) were analyzed by one way ANOVA and

the post-hoc analysis was performed with the Tukey-HSD multiple range test with the significance level set at 0.05.

Submandibular Gland Acinar Cell Preparation and Primary Cultures

A pair of glands from one adult female rat (150-200 g of body weight) was routinely used in each experiment. The submandibular gland cells were prepared by enzymatic dispersion (Fleming *et al.*, 1980; Quissell and Redman, 1979). Glands were rinsed in Ca^{2+} -and Mg^{2+}-free Hanks' balanced salt solution (HBSS), and kept on ice. The glandular tissue was minced into small pieces with scissors, rinsed twice in the same solution and transferred to 25 ml of dispersion medium [HBSS containing 75 U/ml of collagenase (type CLS 2; Worthington Biochemical Corp., Freehold, NJ) and 0.1-0.2 mg /ml hyaluronidase (type 1-S; Sigma Chemical Co., St Louis, Mo] in a siliconized and sterile Erlenmeyer flask, and placed in a Dubnoff-type metabolic shaker at 37° C, and shaking at 110 rpm. At 15-min intervals the tissue was dispersed by pipetting up and down with a sterile fire polished Pasteur pipette. After approximately 1 hr, the resulting acinar cell suspension was washed twice in HBSS by centrifugation at ~400 g for 30 sec, and resuspended in 25 ml of incubation medium (F-12, 10% FCS). Any large, undispersed tissue clumps were allowed to settle out for ~30 sec and removed. Cell viability was estimated by trypan blue dye exclusion, and was always > 90%. Aliquots of 1 ml were transferred to individual wells of collagen coated Multidish

6 well plates (Nunclon Delta; Denmark) in a final volume of 2 ml of incubation medium and then preincubated at 37° C, 5% CO_2. After 1 h of preincubation, the cells were treated with IPR (10^{-5} M final concentration), dibutyryl-cAMP (10^{-3} M), β-estradiol (10^{-8} M), or dexamethasone (10^{-6} M), and incubated for ~18-22 h. Control cells were not treated with the drugs. After the incubation period, the cells were collected and proteins for Western blot analyses were prepared by lysing the cells in RIPA lysis buffer (150mM NaCl, 1.0 % NP-40, 0.5 % DOC, 0.1 % SDS, 50 mM Tris, pH 8.0). Total RNA for RT-PCR analyses were prepared as described previously.

SDS-polyacrylamide gel electrophoresis of submandibular gland proteins, electroblotting, and immunostaining of the electroblots

Protein electrophoresis in 12 % polyacrylamide slab gels, electrotransfer of the proteins to nitrocellulose paper, and immunostaining of the blots were performed following standard protocols as described in Harlow and Lane (1988). A rabbit anti-LM antiserum (1:1000) as the primary antibody (Naito, 1981), and goat anti-rabbit IgG alkaline phosphatase-conjugated (1:7000) as the second antibody, were used.

RT-PCR Analysis of Cystatin S gene Expression in Primary Cultures of Rat Submandibular Glands

Cystatin S gene expression in control and IPR-treated cells from primary cultures of submandibular glands was analyzed by reverse transcriptase-PCR amplification (RT-PCR) of rat cystatin S mRNA using a GenAmp kit (Perkin Elmer Cetus; Branchburg, NJ) following the manufacturers instructions. Reverse transcription for 15 min at 70° C, followed by 1 cycle at 95 ^{0}C, and 25 cycles for 1 min at 95 ^{0}C and 1 min at 60 ^{0}C. In order to radioactively label the PCR products, 5 μl of PCR buffer containing ^{32}P-αATP (0.1 μM) were added to each reaction tube during the last 5 cycles of amplification. As a template, 250 ng of total RNA were used. As a positive control, 100 ng of total RNA from the submandibular gland of an adult female rat treated with IPR were used. The upstream primer 5'-GATATCATGAACCACGAAAGA-3' that primes at position +152 to +172 and the down stream primer 5'-CTAGGTCACTTTCTGGGTGGC-3' priming at position +5024 to +5044 of the genomic DNA sequence, amplify a 296 bp DNA fragment from the cDNA produced from the cystatin S mRNA. This distinguishes the PCR product from any potential amplification from the genomic DNA, that would produce a 4892 bp fragment. The amplification products were analyzed in 5% polyacrylamide-urea denaturing gels, transferred onto GeneScreen Plus membranes and analyzed in the PhosphorImager (Molecular Dynamics) as described previously.

CHAPTER III

EFFECT OF SYMPATHECTOMY ON THE ISOPROTERENOL-INDUCED EXPRESSION OF THE CYSTATIN S GENE IN RAT SUBMANDIBULAR GLANDS

INTRODUCTION

Both branches of the autonomic nervous system innervate the major salivary glands of the rat and regulate the volume and composition of saliva (Emmelin, 1964). In adult animals, stimulation of cholinergic and α-adrenoreceptors elicits the formation of a moderate amount of saliva rich in electrolytes, whereas stimulation of β-adrenoreceptors causes an extensive degranulation of both acinar and granular duct cells, and secretion of small amounts of saliva rich in proteins (Garret *et al.*, 1991; Garrett, 1987; Martinez *et al.*, 1975). Although greater flow rates are achieved than during sympathetic nerve activation, no morphological evidence has been found that parasympathetic stimulation causes any acinar or granular duct degranulation (Garret *et al.*, 1991).

It has long been known that the size of salivary glands is influenced by the autonomic nervous system, and that in adult rats prolonged electrical stimulation

of the sympathetic branch of the autonomic nervous system via the superior cervical ganglion (SCG) causes enlargement of salivary glands by increasing both cell size and cell number (Muir *et al.*, 1975; Schneyer, 1972).

In addition to the classical neurotransmitters norepinephrine and acetylcholine, several neuropeptides have been identified that appear to play a significant role in the regulation of the submandibular gland metabolism (Ekström, 1987). Although the roles of neuropeptides in neurotransmission are poorly defined, it is assumed that they act as co-transmitters or neuromodulators (Rao and Landis, 1993). Sympathetic neurons produce a number of neuropeptides, including neuropeptide Y (NPY) (Jarvi *et al.*, 1986; Lundberg *et al.*, 1982b), galanin, enkephalins (Domeij *et al.*, 1991; Happola *et al.*, 1987), calcitonin-gene related peptide (CGRP), and vasoactive intestinal peptide (VIP) (Elfvin *et al.*, 1993). It has been proposed by several investigators that the neurotransmitter repertoire expressed by mature neurons is often correlated with the particular peripheral target and that target tissues instruct the innervating neurons to synthesize the specific neurotransmitters and neuropeptides appropriate for the target, or suppress the synthesis of those that are unnecessary (Klimaschewski *et al.*, 1996; Rao and Landis, 1993; Schotzinger *et al.*, 1994). For example, neurons in the rat superior cervical ganglion that innervate three different targets (submandibular gland, eyes, and pineal gland), differ in size, number, location within the ganglion and in their neuropeptide content (Luebke and Wright , 1992).

In adult animals, denervation or pharmacological blockage of receptor action lead to a supersensitive response and upregulation of receptor numbers, not only in the submandibular gland (Arnett and Davis, 1979; Pointon and Banerjee, 1979) but also other organs like heart and kidney (Yamada *et al.*, 1980; Yamada *et al.*, 1986). Surgical removal of the superior cervical ganglion from adult rats results in supersensitivity of the submandibular gland to the β-adrenergic sialogogic effects of norepinephrine and IPR (Ekström, 1980; Emmelin *et al.*, 1965; Perec *et al.*, 1975; Perec *et al.*, 1973; Stefano and Perec, 1981).

Catecholamine stores in sympathetically innervated organs are depleted rapidly after a single administration of reserpine, and the depletion is almost complete (>95%) within the first day. As a result, in salivary glands there is a decreased concentration of norepinephrine at the synapse (Bahouth, 1992), a marked increase in adrenergic receptors (Bylund *et al.*, 1982), and also supersensitivity to IPR (Bylund *et al.*, 1981; Martinez *et al.*, 1975).

As discussed in Chapter II, the expression of the cystatin S gene induced by IPR in the submandibular gland of adult rats seems to be regulated through the β_1-adrenergic receptor-cAMP-PKA pathway since the effect of IPR is blocked by specific β_1-, but not by β_2-adrenergic antagonists (Bedi, 1991a). It is not known however, if this pathway regulates directly or indirectly the expression of the cystatin S gene and if other factor(s) participate in this regulation. Since submandibular glands sympathetically denervated (surgically or chemically by

reserpine treatment) become supersensitive to the action of β-adrenergic agonists, one would expect an overexpression of the cystatin S gene upon IPR treatment if the gene is regulated directly by cAMP-mediated mechanisms. It has been demonstrated that isolated acini from rat submandibular glands are responsive to β-adrenergic agonists (Bradbury and McPherson, 1988; Fleming *et al.*, 1980), and that acinar cells *in vitro* respond to IPR by increasing intracellular levels of cAMP (Rajakumar and Scarpace, 1994). It is not known, however, if an increase of intracellular cAMP by itself is sufficient for cystatin S gene expression, or if this induction requires *de novo* protein synthesis (such as specific transcription factors). If an increase of intracellular cAMP is sufficient for the induction of cystatin S gene expression in acinar cells, one would expect the gene to be expressed *in vivo* in response to IPR even in the presence of protein synthesis inhibitors, and *in vitro* in acinar cells treated with IPR.

This chapter describes experiments designed to test these hypotheses, by analyzing the effect of sympathectomy of the submandibular gland of adult female rats, by removing the superior cervical ganglion (bilaterally or unilaterally), or by chemical denervation by treatment with reserpine, on the IPR-induced expression of the cystatin S gene. In addition, the effect of the inhibition of protein synthesis by cycloheximide *in vivo* on the IPR induced expression of the cystatin S gene was analyzed.

Methods

The methods used for surgical and chemical sympathectomy, IPR treatment of the animals, Northern blots, quantitative and statistical analyses of the data were described in the General Methods Chapter.

The effect of bilateral sympathectomy on the IPR-induced expression of the cystatin S gene was analyzed in submandibular glands of female adult rats, treated with IPR 3 days and 14 days after surgery. Based on the initial results of bilateral sympathectomy experiments, quantitative analyses of the effect of surgical bilateral or unilateral sympathectomy on IPR-induced cystatin S gene expression were performed in adult female rats 14 days after surgery.

RESULTS

Standard Curve for Linearity of Signal Detection on the PhosphorImager

The linearity of the signal detected in the PhorphorImager was analyzed by quantitation of dot blot hybridization of increasing amounts of total RNA from the submandibular glands of IPR-treated female rats, hybridized to a cystatin S specific riboprobe. The standard curve in **Figure 3-1** shows the linearity of the signal for total RNA amounts from 0 to 1.5 μg. The linear regression analysis of the data yielded an r^2 of 0.991.

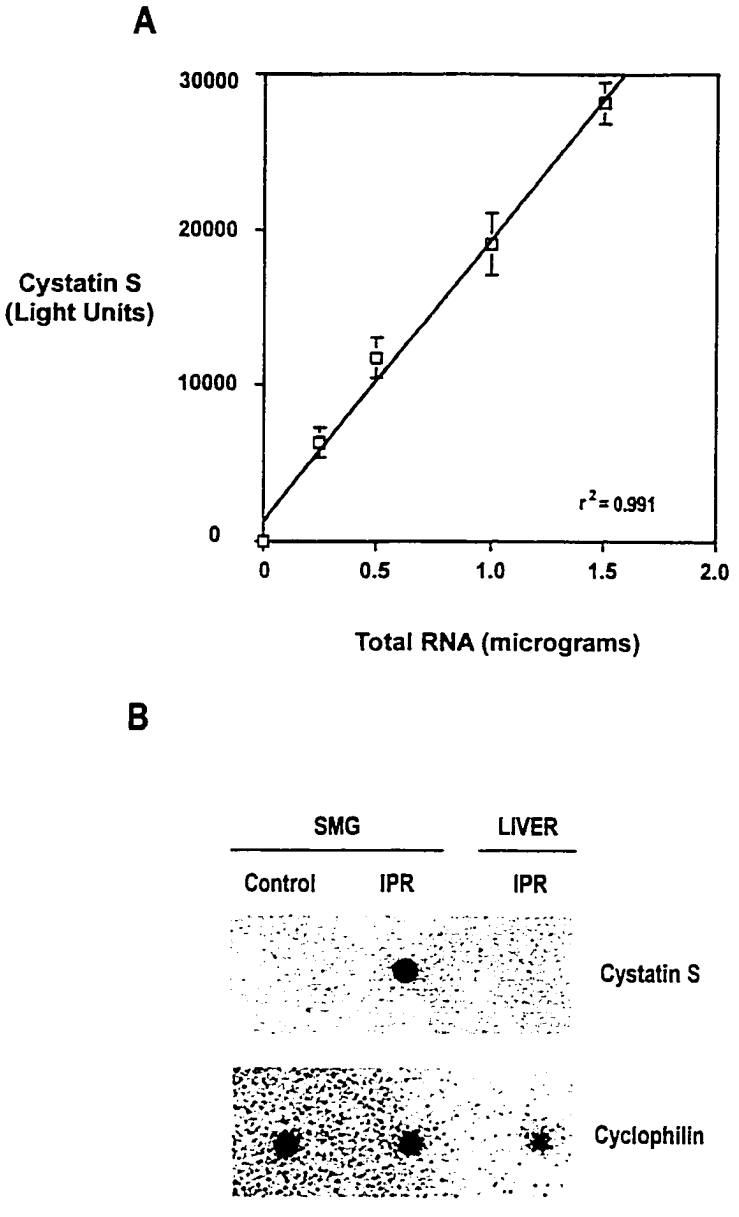


Figure 3-1. Standard curve for linearity of signal detection on the PhosphorImager. A. Dot blot analyses of increasing amounts of total RNA from submandibular glands of IPR-treated adult female rats. Each point represent the mean +/- standard error of the mean (SEM) of 5 different samples. **B.** Dot blot hybridization of 1.5 μg of total RNA showing the specificity of the cystatin S riboprobe.

IPR-Induced expression of the cystatin S gene

As previously demonstrated (Shaw *et al.*, 1990), cystatin S mRNA was not detected by Northern blot hybridization of total RNA in submandibular glands of adult female rats. However, high levels of the message were present in the glands, 24 hours after a single injection of IPR (**Figure 3-2**). The level of cyclophilin mRNA was not affected by IPR treatment.

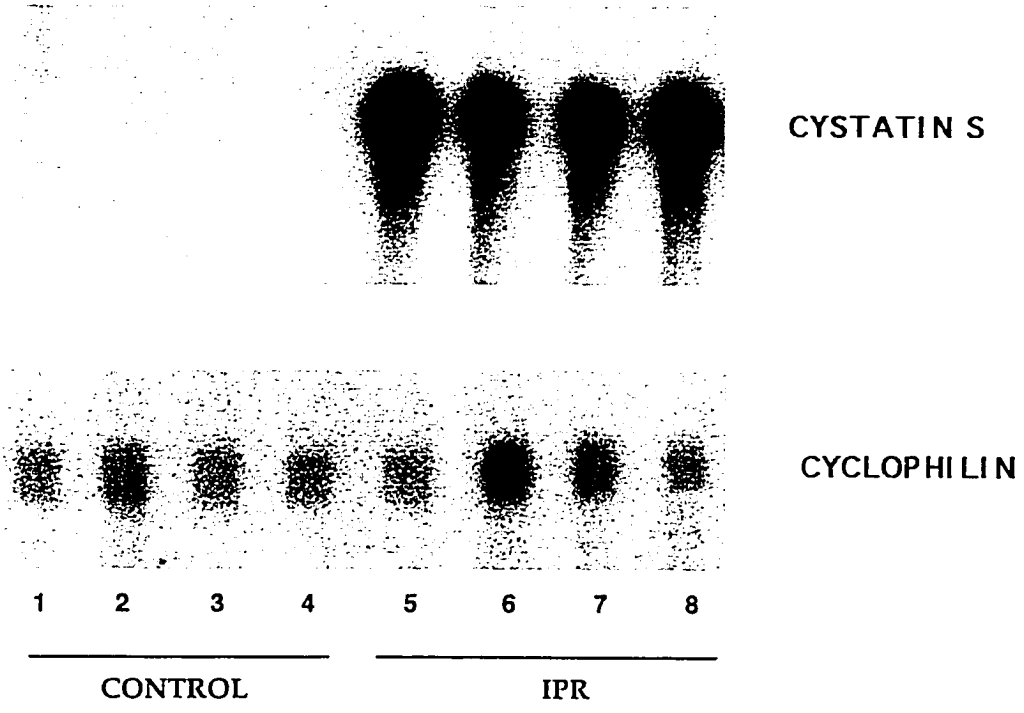


Figure 3-2. Northern blot analyses of cystatin S mRNA from submandibular glands of adult female rats untreated or treated with IPR. Total RNA was extracted from submandibular glands 24 hr after IPR treatment, electrophoresed and hybridized as described in General Methods. One microgram of total RNA was loaded into each well. Duplicate filters were hybridized for cyclophilin. Lanes 1-4, Control (untreated) rats. Lanes 5-8, IPR-treated rats.

Effect of bilateral sympathectomy on the IPR-induced expression of the cystatin S gene in the rat submandibular gland

Bilateral sympathectomy of adult rats caused a reduction in the levels of cystatin S mRNA induced by IPR in submandibular glands, compared to glands of sham-operated, IPR-treated animals. **Figure 3-3** depicts the results of Northern blots indicating that the steady-state level of cystatin S mRNA was reduced as early as 3 days post sympathectomy (**Figure 3-3 A**). The levels of cystatin S mRNA in IPR-treated submandibular glands 14 days after sympathectomy were drastically reduced compared to those in sham-operated IPR-treated animals (**Figure 3-3 B**). On the basis of these results, more extensive studies were carried out choosing the 14 day time point since it showed the most dramatic reduction in IPR-induced cystatin S mRNA (**Figure 3-3**). The amount of cystatin S mRNA in the submandibular glands of female adult rats, bilaterally sympathectomized 14 days before one single injection of IPR, was reduced to approximately 47 % of that of submandibular glands of sham-operated animals (**Figures 3-4 & 3-5,** $p = 0.029$, $n = 4$).

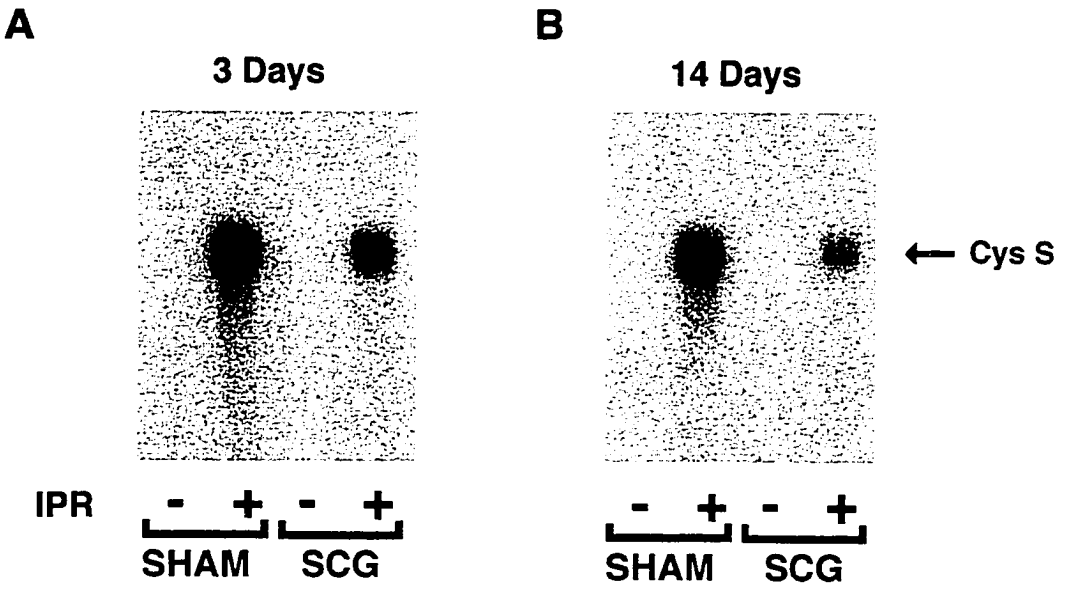


Figure 3-3. Northern blot analyses of cystatin S mRNA from submandibular glands of sham-operated (Sham) and bilaterally sympathectomized (by removing the superior cervical ganglion, SCG) adult female rats untreated or treated with IPR, 3 or 14 days post denervation. A. Three days post sympathectomy. **B.** Fourteen days post sympathectomy.

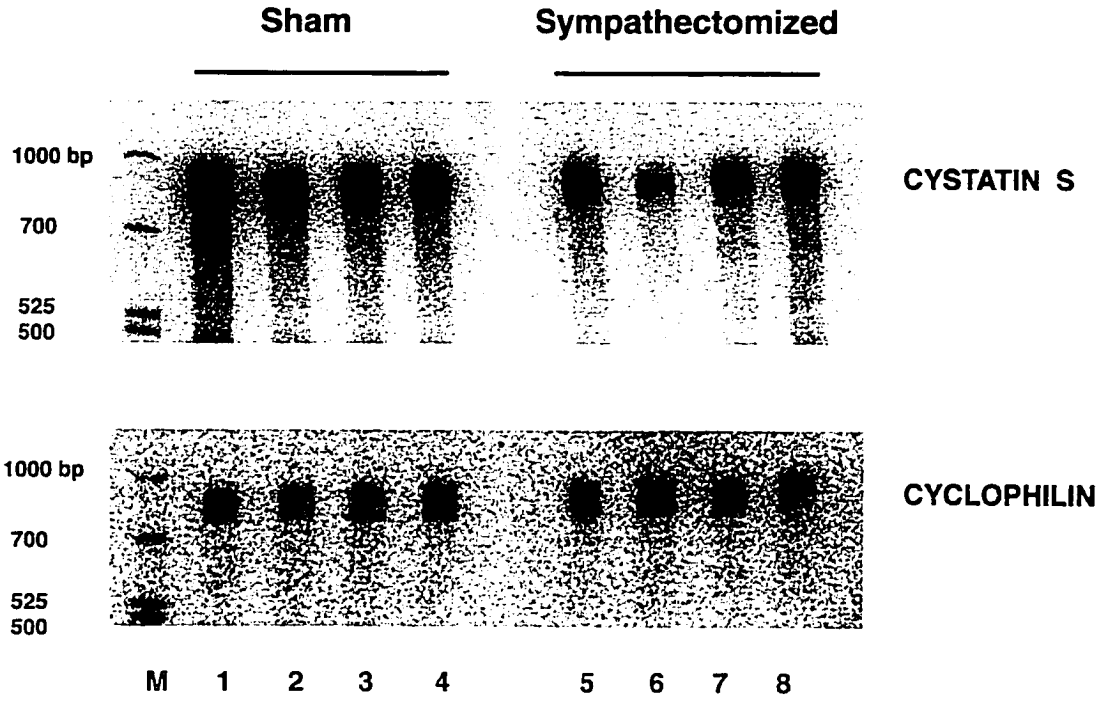


Figure 3-4. Northern blot analyses of cystatin S mRNA from submandibular glands of sham-operated and bilaterally sympathectomized adult female rats treated with IPR 14 days after denervation. M, molecular weight markers; lanes 1-4, four sham-operated, adult female rats treated with IPR. Lanes 5-8, four bilaterally sympathectomized adult female rats treated with IPR.

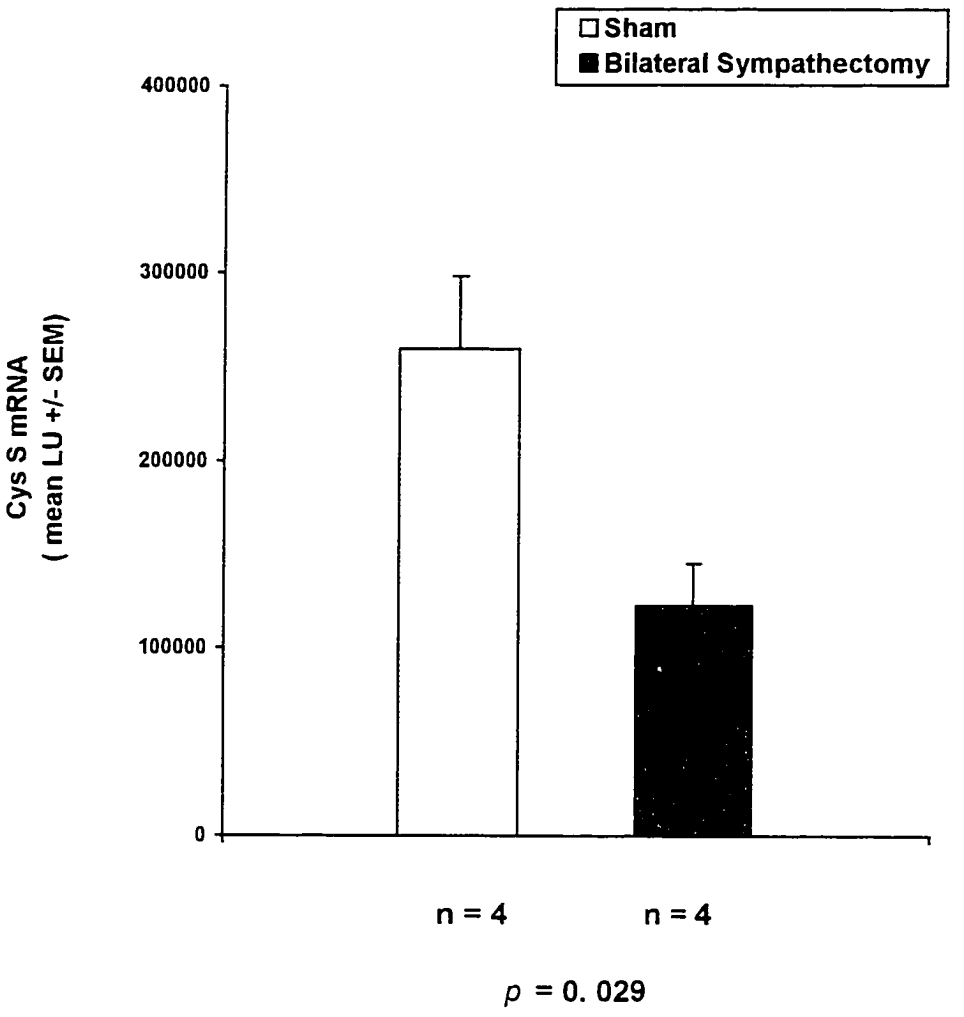


Figure 3-5. Quantitation of cystatin S mRNA levels in submandibular glands of sham-operated and bilaterally sympathectomized IPR-treated rats. Quantitative analyses of the Northern blots in Figure 3-4 were performed using the PhosphorImager (Molecular Dynamics). The data are expressed as Light Units (Mean LU +/- SEM) and analyzed by a *t*-test for independent samples.

Effect of unilateral sympathectomy on the IPR-induced expression of the cystatin S gene in the rat submandibular gland

Unilateral sympathectomy experiments were performed in which one intact gland served to assess the response of the cystatin S gene to IPR, while the denervated gland was used to analyze the effect of sympathectomy upon IPR-induced expression of the rat cystatin S gene. In Northern blots of untreated unilaterally sympathectomized rat submandibular glands, cystatin S mRNA was undetectable (**Figure 3-6**). These data demonstrate that the denervation process itself does not induce the expression of the cystatin S gene. When IPR was administered to rats 14 days after unilateral sympathectomy, the concentration of cystatin S mRNA in denervated submandibular glands was reduced compared to the intact gland (**Figure 3-6**).

Quantitative analyses was then performed in a group of eight adult female rats, treated with a single injection of IPR, 14 days after unilateral sympathectomy. The levels of cystatin S mRNA in sympathectomized submandibular glands (right glands) were approximately 56 % of those in intact (left glands) submandibular glands (**Figures 3-7 & 3-8,** $p = 0.005$, n = 8).

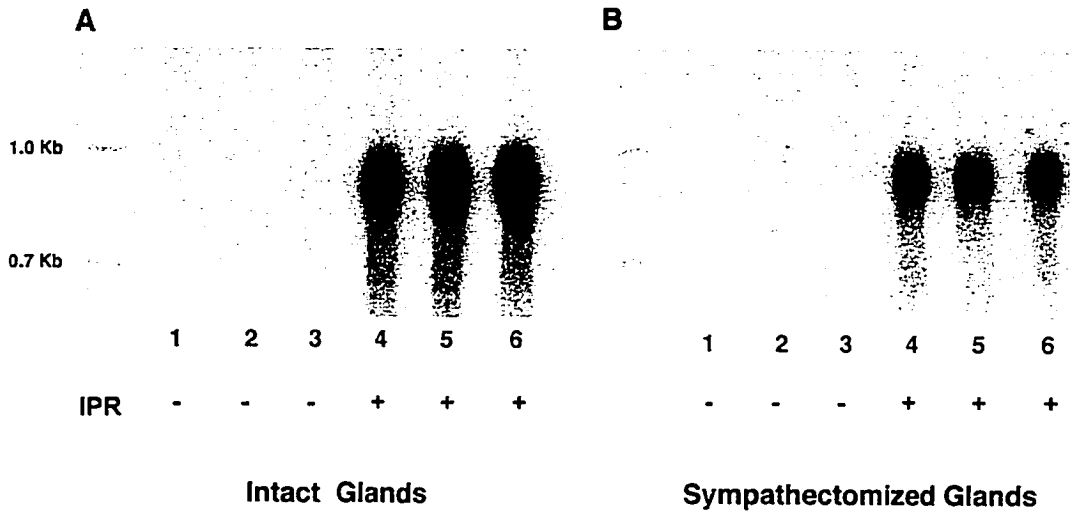


Figure 3-6. Northern blot analyses of cystatin S mRNA from submandibular glands of unilaterally sympathectomized adult female rats, untreated and treated with IPR.
A. Total RNA from left glands (intact, no sympathectomy). **B.** Total RNA from the corresponding right sympathectomized submandibular glands.

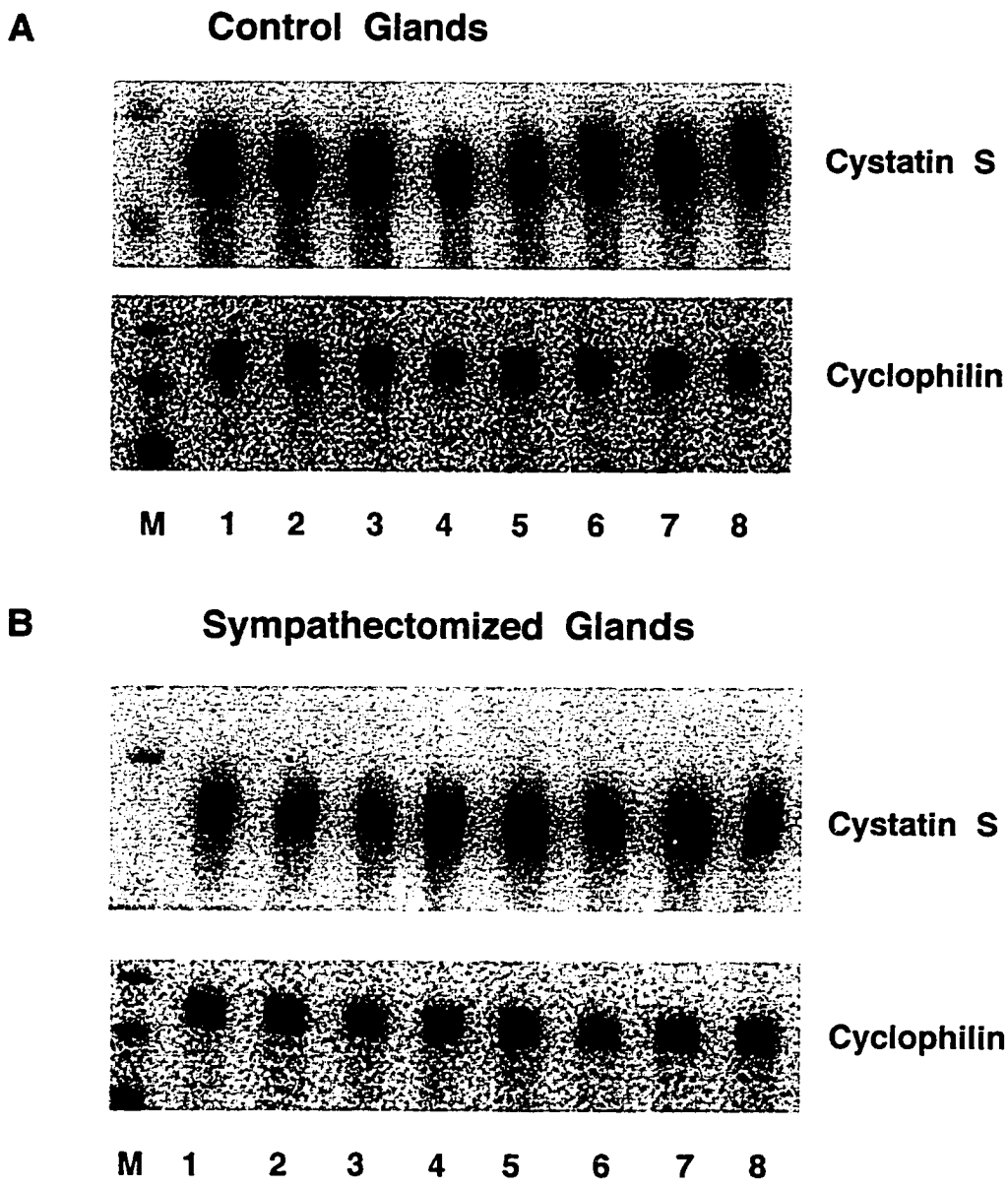


Figure 3-7. Northern blot analyses of cystatin S mRNA from submandibular glands of unilaterally sympathectomized adult female rats, treated with IPR. A. Total RNA from left (non-sympathectomized) glands. M, molecular weight markers; Lanes 1-8, eight unilateral sympathectomized adult, female rats. **B.** M, markers; lanes 1-8, total RNA from the corresponding right (sympathectomized) glands.

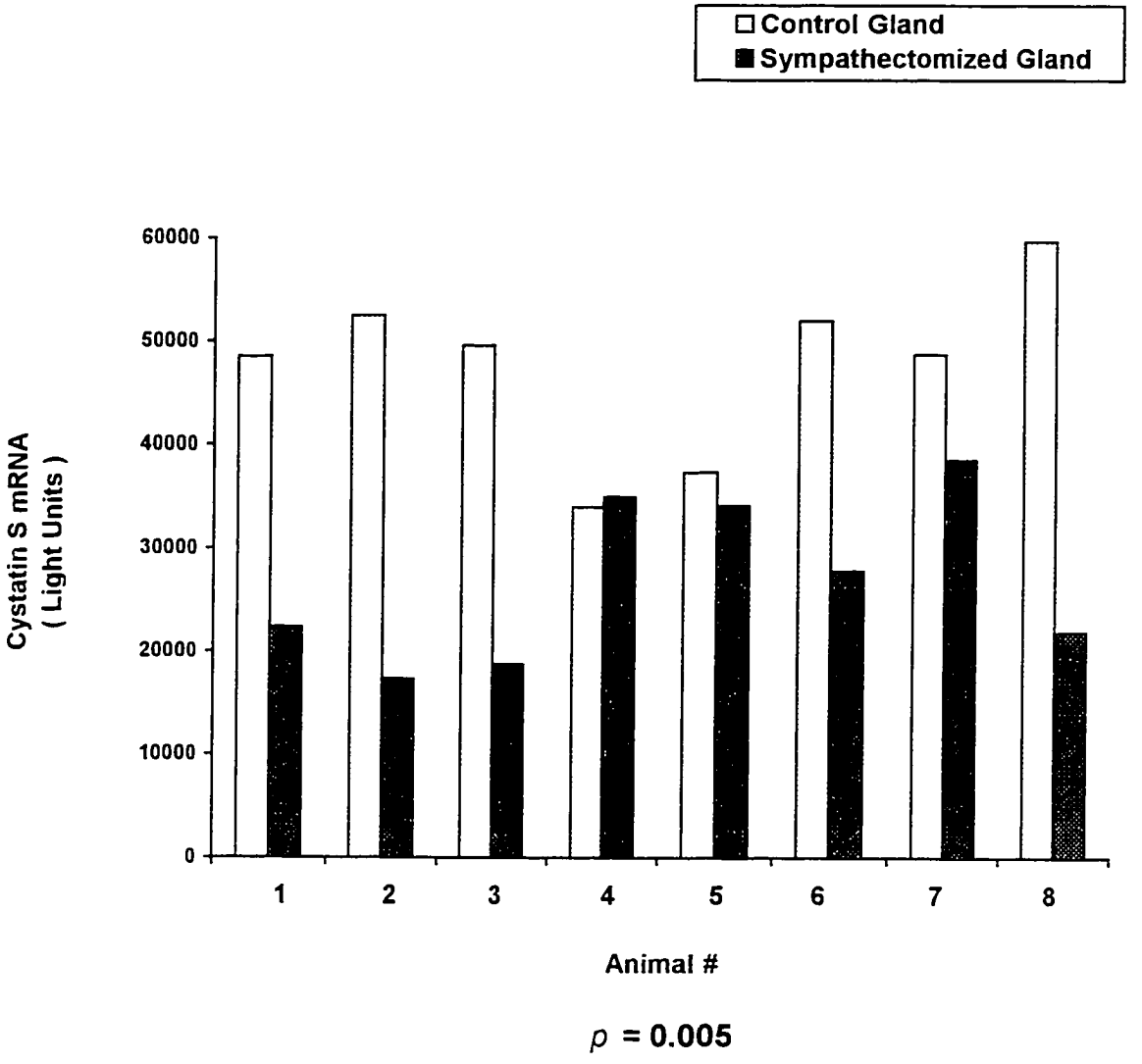


Figure 3-8. Effect of unilateral sympathectomy on the IPR-induced expression of the cystatin S gene in rat submandibular glands. Quantitative analyses from eight animals represented in Figure 3-7 were performed using the PhosphorImager (MolecularDynamics). The data are expressed as Light Units and analyzed by a *t*-test for paired samples. Each pair of bars represent the control and sympathectomized glands from the same animal.

Unilateral or bilateral sympathectomy caused no significant change in the relative weight of the submandibular glands. Rats killed 24 hours after the administration of a single dose of IPR had an average of 12% heavier submandibular glands, irrespective of whether the animals were unilaterally or bilaterally sympathectomized, compared to untreated rats. This increase, however, was not statistically significant (**Table 2**).

Table 2

Effect of bilateral or unilateral sympathectomy on the weight of rat submandibular glands

IPR was injected 14 days after denervation as described in Materials and Methods. The data are reported in mg tissue per 100 g body weight. Each value is the mean and standard deviation of the mean (in parentheses). The differences between the paired means of the right and left glands were statistically not significant.

	CONTROL		IPR	
	Gland		Gland	
Treatment	Right	Left	Right	Left
Shams	93.3 (13.3) n = 5	94.3 (15.5) n = 5	105.3 (15.5) n = 6	104.6 (17.9) n = 6
Bilateral sympath.*	82.1 (3.9) n = 3	89.4 (3.4) n = 2	94.2 (20.9) n = 4	102.6 (17.3) n = 4
Unilateral sympath.ð	89.8 (8.2) n = 9	95.0 (11.2) n = 9	91.1 (9.6) n = 11	96.8 (12.2) n = 11

* The superior cervical ganglion was removed from both sides.

ð The superior cervical ganglion was removed from the right side only.

The submandibular glands from unilaterally sympathectomized rats were examined microscopically. **Figure 3-9** are representative micrographs illustrating the changes seen. Sympathectomy caused no conspicuous structural alterations in the submandibular glands. However, the acini in the submandibular glands of sympathectomized untreated rats were somewhat smaller, condensed, and contained less secretory material (**Figure 3-9 A, B, C**). As described previously, the glands of rats which received IPR 24 hours earlier, had enlarged acinar cells that were filled with secretory material (**Figure 3-9 D, E, F**). Hypertrophy appeared to be less pronounced in the acini of submandibular glands from animals which were unilaterally sympathectomized (right gland) 14 days prior to the administration of IPR (**Figure 3-9 F**). There were no obvious changes in the frequency or structure of the ducts in the glands of sympathectomized animals.

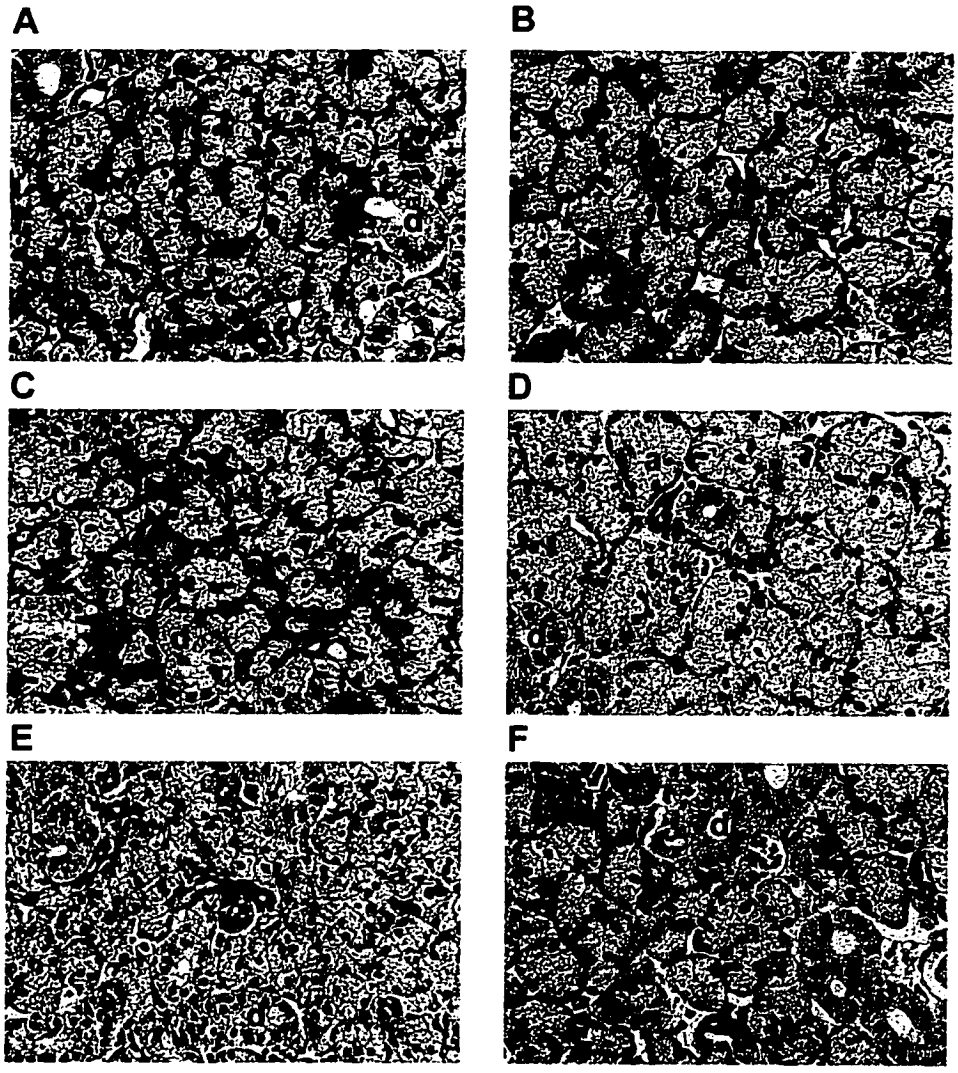


Figure 3-9. Submandibular glands of sham and unilaterally sympathectomized adult female rats stained with Hematoxylin and Eosin (H & E). Acini (a), striated ducts (d). (X125). **A.** Submandibular gland from a sham-operated untreated animal. **B.** Non-sympathectomized submandibular gland from untreated rat. **C.** Sympathectomized submandibular gland from the same untreated rat. **D.** Submandibular gland from a sham-operated IPR-treated rat. **E.** Non-sympathectomized submandibular gland from IPR-treated rat, showing similar degree of enlargement of acini (a) as in sham-operated animals in D. **F.** Sympathectomized submandibular gland from the same IPR-treated rat as in E, showing enlargement of acini (a) in response to IPR as compared to C. These results are typical of those observed in all eight animals

Effect of chemical sympathectomy by reserpine treatment on the IPR-induced expression of the cystatin S gene in the rat submandibular gland

In addition, IPR-induced cystatin S gene expression was analyzed in submandibular glands from animals treated with reserpine, a drug that depletes neurotransmitters from sympathetic nerve terminals. Cystatin S mRNA levels in the submandibular glands of adult rats treated with a single dose of reserpine for each of 2 days 24 hours prior to IPR injection were approximately 30 % of those in non-reserpine, IPR- treated rats (**Figures 3-10 & 3-11**, $p = 0.024$, n = 5).

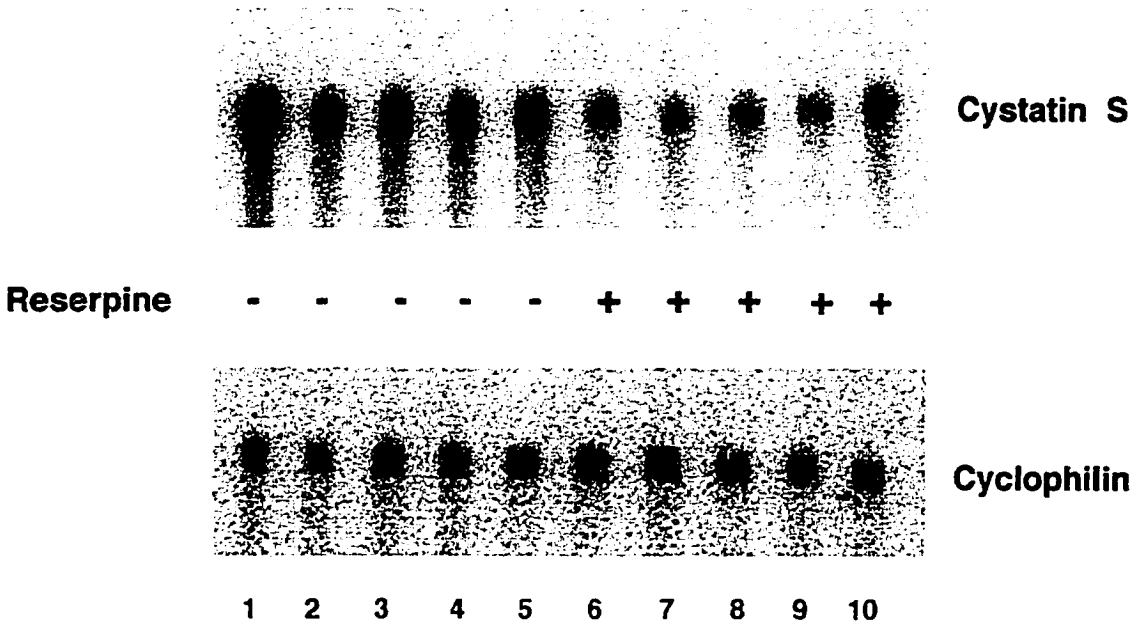


Figure 3-10. Northern blot analyses of cystatin S mRNA from submandibular glands of control or reserpine-treated female adult rats treated with IPR. Lanes 1-5, total RNA from glands of five animals treated with a single injection of IPR. Lanes 6-10, total RNA from glands of adult females rats, treated with a single daily injection of reserpine for two days, and 24 hour later with a single injection of IPR.

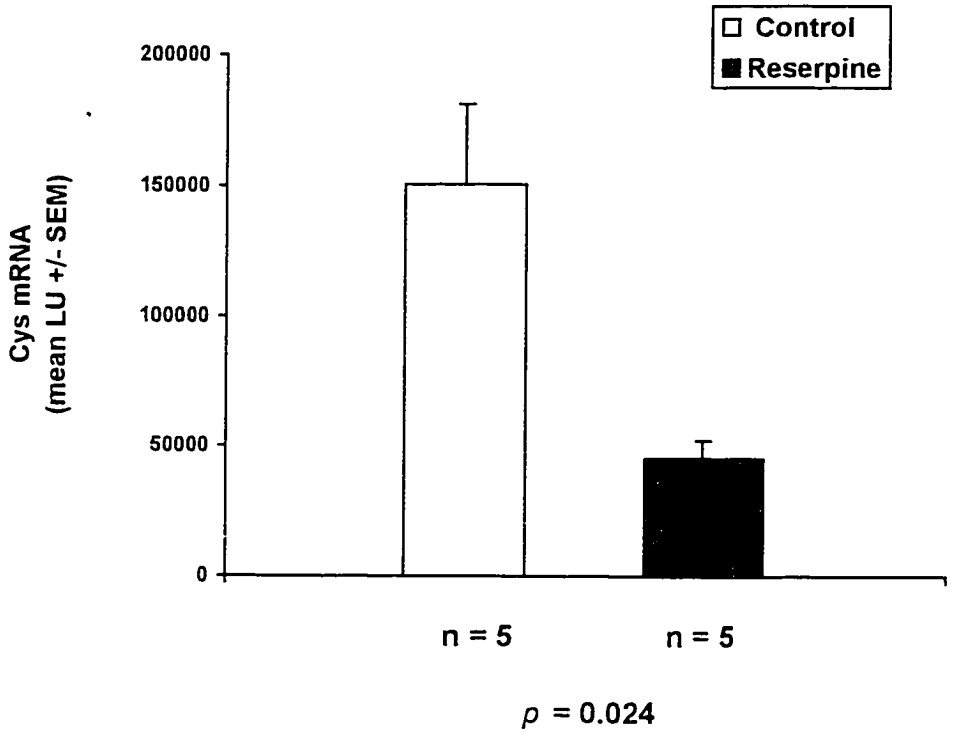


Figure 3-11. Quantitation of cystatin S mRNA levels induced by IPR in submandibular glands of control and reserpine-treated rats. Control animals were treated with a single injection of IPR as described in General Methods. Reserpine treated animals were given a single dose of reserpine (0.5 mg/kg body weight) for each of two days; then, 24 hours later, a single dose of IPR. The data are expressed in Light Unit (Mean +/- SEM) and analyzed by a *t*-test for independent samples.

Submandibular glands from reserpine treated animals were also examined microscopically. A representative experiment (two animals) showed that in control animals (no reserpine), IPR dramatically enlarged the acini compared to untreated glands (**Figure 3-12 A & B**). The acini in reserpine plus IPR-treated animals were slightly smaller, demonstrating however, a hypertrophic response (**Figure 3-12 C & D**). There were no obvious changes in the frequency or structure of the ducts. Reserpine caused no noticeable structural alterations in the submandibular glands under these experimental conditions.

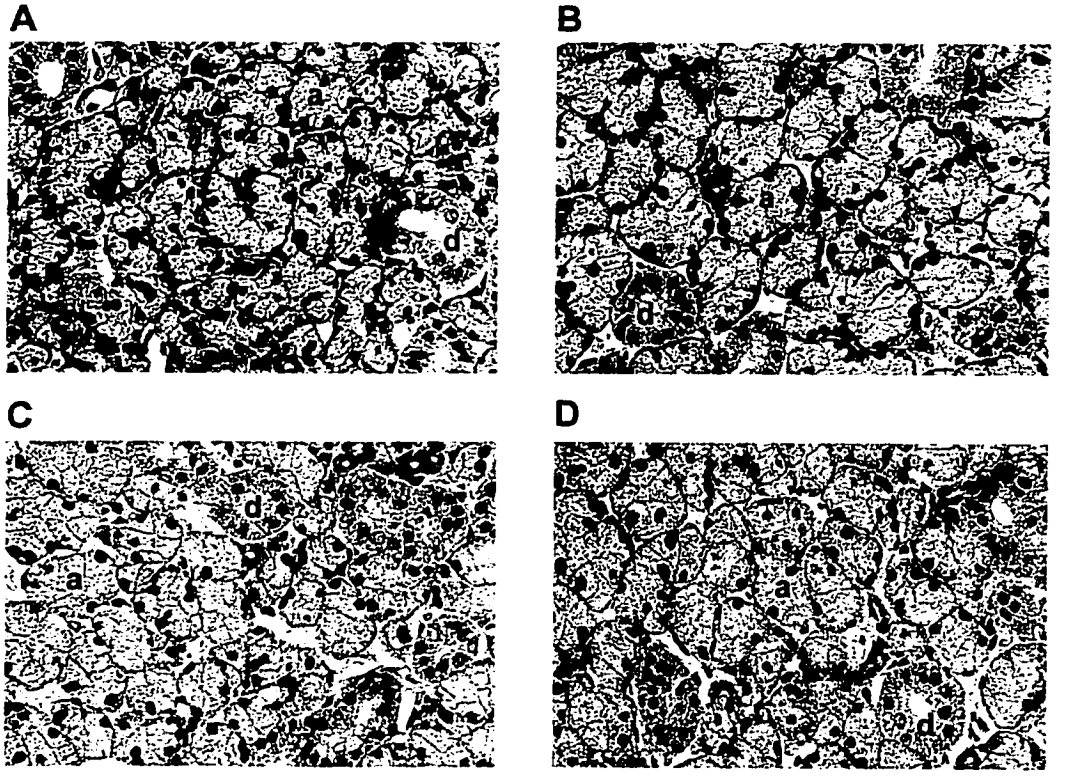


Figure 3-12. Submandibular glands of IPR-treated, and reserpine and IPR-treated adult female rats stained with H & E. Acini (a), striated or granular convoluted tubules (d). X125. **A.** Submandibular gland from an untreated animal. **B.** Submandibular gland from an IPR-treated animal showing hypertrophy of the gland. **C and D.** Submandibular glands from two different animals treated with reserpine for 2 days and then with IPR, showing a similar enlargement as the gland in B. These results are typical of those observed in all five animals.

Effect of protein synthesis inhibition on the IPR-induced expression of the cystatin S gene in the rat submandibular gland

The effect of cycloheximide, a protein synthesis inhibitor, on IPR-induced expression of the cystatin S gene in the submandibular glands of adult female rats was analyzed by Northern blot hybridization. Cystatin S mRNA was clearly detectable 6 hours after a single injection of IPR (**Figure 3-13, lanes 3, 4**). Cycloheximide alone did not induce detectable expression of the cystatin S gene (**Figure 3-13, lanes 1, 2**). However, simultaneous treatment with cycloheximide and IPR completely eliminated IPR-induced expression of the cystatin S gene (**Figure 3-13, lanes 5, 6**). These results clearly indicate that protein synthesis is required for IPR induction of cystatin S gene expression. No significant changes were observed in the expression of cyclophilin, suggesting that cycloheximide had no general inhibitory effect on transcription in the rat submandibular gland (**Figure 3-13**).

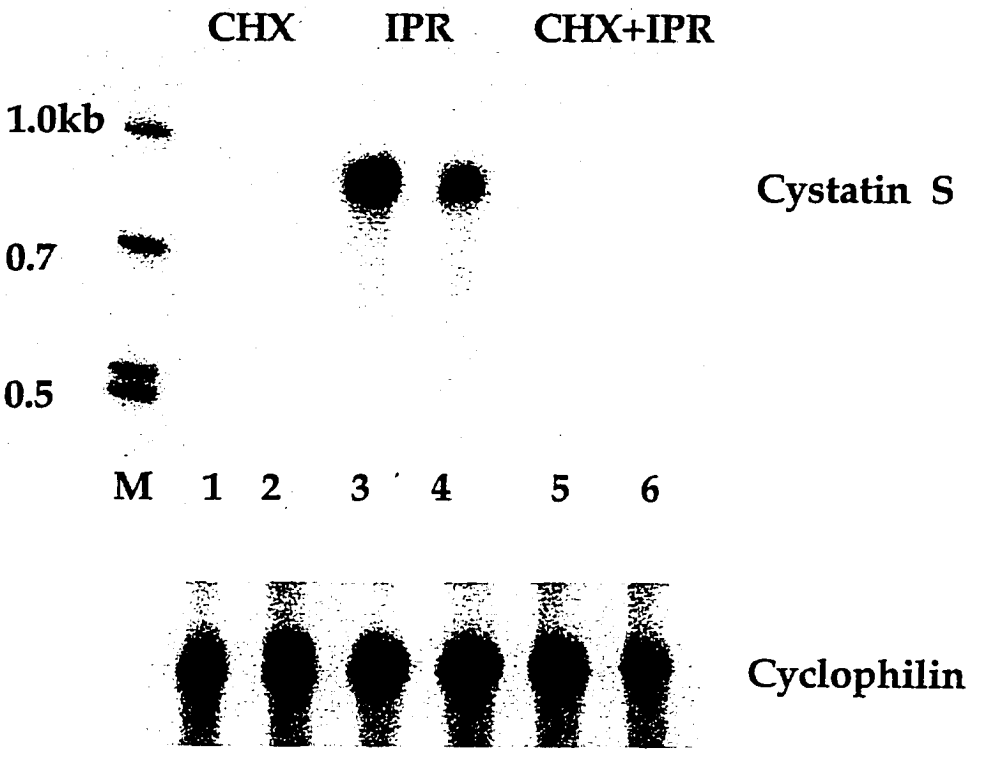


Figure 3-13. Northern blot analyses of the effect of cycloheximide on IPR-induced cystatin S gene expression. Five µg of total RNA extracted from submandibular glands were loaded into each well. Lanes 1 and 2 are from two adult female rats treated with cycloheximide alone, lanes 3 and 4 are from two female rats treated with IPR. Lanes 5 and 6 are from two female rats treated with cycloheximide and IPR simultaneously.

Discussion

In adult animals, denervation of adrenergic target tissues leads to compensatory upregulation of α- and β-adrenergic receptors and to supersensitive responses to adrenergic agonists. This supersensitivity has been documented, for example, in rat cerebral cortex (Sporn *et al.*, 1976), rat urinary bladder (Ekström, 1979), rat pineal gland (Weiss *et al.*, 1980), rat parotid gland (Schneyer *et al.*, 1988), and in smooth muscles of rabbit iris (Abdel-Latif *et al.*, 1995). Surgical or chemical sympathetic denervation in adult rats results in supersensitivity of the submandibular gland to the β-adrenergic sialogogic effects of norepinephrine and IPR (Ekström, 1980; Emmelin *et al.*, 1965; Perec *et al.*, 1975; Perec *et al.*, 1973; Pointon and Banerjee, 1979; Stefano and Perec, 1981).

Binding studies have demonstrated that the increase in the β-adrenergic response in sympathectomized rat submandibular glands is paralleled by an increase in the density of the β_1-adrenergic receptors in membranes prepared from the glands, with no change in binding affinity (Arnett and Davis, 1979); Bahouth, 1992; Pointon and Banerjee, 1979). The supersensitivity of the submandibular gland was also illustrated by the increase in intracellular cAMP in response to IPR in cells from submandibular glands of rats previously treated with reserpine as compared with cells from glands of control animals as reported by Bylund *et al.*, 1981. Basal cAMP levels were similar between control (1.4+/- 0.3 pmol/mg) and

reserpine treated glands (2.0 +/-0.6 pmol/mg). However, the increase in intracellular cAMP in response to IPR was markedly accentuated in glands from reserpine treated rats (79-fold increase) compared with control glands (13-fold increase) (Bylund *et al.*, 1981). It has also been demonstrated that the submandibular gland remains supersensitive to norepinephrine 6 weeks after sympathetic denervation (Asking and Ekström, 1979).

The results of the experiments described in this chapter are somewhat unexpected. If, cystatin S gene expression is regulated directly by the β_1-adrenergic receptor pathway, one would have expected one of the following results: (1) Overexpression of the cystatin S gene in response to IPR in sympathectomized animals since β_1-adrenoreceptors are upregulated upon surgical sympathectomy or reserpine treatment, and submandibular glands become supersensitive to β-adrenergic agonists. (2) Alternatively, if the intracellular concentration of cAMP reached in the submandibular glands of non-denervated rats upon treatment with IPR is sufficient to produce the maximal level of expression of the cystatin S gene, it would be expected that in the denervated glands (which are supersensitive to IPR), a further increase in the intracellular concentration of cAMP in response to IPR would not produce any significant change in the levels of cystatin S gene expression. However, both bilateral and unilateral sympathetic denervation reduced the levels of cystatin S gene expression in response to IPR (**Figures 3-4, 3-5, 3-7 & 3-8**), and a comparable effect was

observed in rats previously treated with reserpine (**Figures 3-10 & 3-11**). In other words, the expression of the rat cystatin S gene mediated by β_1-adrenergic receptor stimulation was partially blocked by sympathetic denervation and clearly, there is a lack of correlation between the well documented cAMP accumulation in sympathectomized glands (Bylund *et al.*, 1981; Stefano and Perec, 1981) and cystatin S gene expression.

There are several precedents in the literature for similar phenomena. It has been found that the ability of IPR to stimulate ornithine decarboxylase (ODC), a growth related enzyme, in heart, lung and kidney is reduced by neonatal sympathectomy (Hou *et al.*, 1989a). Beta-adrenergic receptors are not coupled directly to ODC gene expression, but rather receptor stimulation initiates the synthesis of new ODC molecules through a cAMP-dependent process mediated by the protooncogene c-*fos*. It has been also demonstrated that sympathetic denervation by 6-hydroxydopamine produces a loss of the ability of stimulated β-adrenergic receptors to induce expression of the protooncogene c-*fos*, resulting in reduced expression of ODC in the rat cerebellum (Wagner *et al.*, 1995). Transcription of a large number of eukaryotic genes including the protooncogene c-*fos*, somatostatin, tyrosine hydroxylase, proenkephalin, vasoactive intestinal peptide, α-chorionic gonadotropin, and phosphoenolpyruvate carboxykinase (PECPK) is activated in response to increased intracellular levels of cAMP, and each of these genes has a common and well characterized short palindromic core

motif 5'-TGACGTCA-3', the cAMP response element (CRE), in their promoter sequence (Borrelli *et al.*, 1992; Lalli and Sassone-Corsi, 1994; Montminy *et al.*, 1990a; Montminy *et al.*, 1990b; Ziff, 1990). The effect of cAMP on gene transcription is usually rapid and does not require newly synthesized protein(s), suggesting that transcriptional modulation by cAMP involves the covalent modification rather than *de novo* synthesis of nuclear factors (Montminy *et al.*, 1990b; Shikama, 1997). Results presented here, however, show that IPR-induced expression of the cystatin S gene in the rat submandibular gland is dependent on newly synthesized proteins, since its expression is completely blocked by simultaneous treatment of the animals with cycloheximide (**Figure 3-13**). Since IPR-induced expression of the cystatin S gene in submandibular glands is dependent on newly synthesized proteins and it does not increase in the glands of sympathectomized rats treated with IPR (which are supersensitive to β-adrenergic agonist) compared to the non-denervated glands (similarly treated with IPR), these results indicate that the cystatin S gene is not regulated directly by the β-adrenergic receptor-cAMP pathway. However, β_1-adrenergic specific antagonists completely block the ability of IPR to induce cystatin S gene expression (Bedi, 1993). Taken together these results suggest that the β_1-adrenergic receptor pathway is necessary but is not sufficient for the full induction of cystatin S gene expression.

There are interesting examples in the rat parotid gland resembling the reduction of the cystatin S gene expression in the submandibular glands of sympathectomized rats in response to IPR: (1) The sympathetic and parasympathetic nervous systems have a regulatory role on proline-rich proteins (PRP) synthesis, since there are significant decreases in the synthesis of PRP in denervated parotid glands of unilaterally sympathectomized, unilaterally parasympathectomized, and double denervated rats (sympathectomized and parasympathectomized). The effect is additive as double denervated glands show changes approximately equal to the combined individual changes of sympathectomy and parasympathectomy (Suares *et al.*, 1997). (2) Maintenance of rats exclusively on a liquid diet results in a reduction in the size of the parotid gland 4 days after introduction of the liquid diet (Hall and Schneyer, 1964). However, reintroduction of a solid diet restores the gland size and also induces a burst in cell proliferation that peaks after 2 days of the dietary change (Schneyer, 1970). This dietary change is accompanied by an increased masticatory activity, and growth responses appear to be mediated by the autonomic nervous system (Hall and Schneyer, 1978). A marked increase in the activity and cell surface levels of the enzyme β1-4 galactosyltransferase (a constituent of the plasma membrane that has a prominent role in mediation of cell growth) is also induced in rat parotid glands when autonomically mediated activity of the gland is increased by a dietary change from all liquid to solid food (Humphreys-Beher and Schneyer,

1987). The same effect is observed following increased acinar cell proliferation of the parotid gland that occurs after chronic exposure of the rats to IPR (Humphreys-Beher *et al.*, 1984). However, removal of either parasympathetic or sympathetic innervation to the parotid gland prior to the dietary change results in a partial inhibition of the increase in β1-4 galactosyltransferase enzymatic activity in parasympathectomized glands (Schneyer *et al.*, 1992b). This picture resembles the observed reduction of IPR-induced expression of the cystatin S gene in sympathectomized submandibular glands, and strongly suggests that the expression of the cystatin S gene in the submandibular gland, β1-4 galactosyltransferase and proline-rich proteins in the parotid gland, and probably other genes regulated through the β-adrenergic receptor pathway (like ODC), require the participation of additional factors from the autonomic (sympathetic and/or parasympathetic) nervous system.

Tissue slices of submandibular glands have been shown to respond to norepinephrine and IPR *in vitro*, as determined by an increase in the levels of cAMP (Bylund *et al.*, 1981; Bylund *et al.*, 1982). Isolated rat submandibular acini have also been also demonstrated to be responsive to α- and β-adrenergic agonists by increased secretion of mucin in response to norepinephrine, IPR (Bradbury and McPherson, 1988; Fleming *et al.*, 1980; Quissell and Redman, 1979; Rajakumar and Scarpace, 1994), dibutyryl cAMP (Quissell *et al.*, 1993), and also to forskolin (Bradbury and McPherson, 1988; Rajakumar and Scarpace, 1994). Responsiveness

of the submandibular gland to IPR *in vitro* has also been documented by the function of stimulatory G proteins (G_s), increased adenylate cyclase activity (Ishikawa *et al.*, 1995), and increased levels of intracellular cAMP (Rajakumar and Scarpace, 1994). However, cystatin S gene expression is not induced *in vitro* in primary cultures of submandibular gland cells in response to IPR (**Figures 1-5 & 1-7**). This lack of inducibility of cystatin S gene expression has also been observed in primary cultures or tissue slices of submandibular glands treated with norepinephrine, forskolin, dibutyryl-cAMP, β-estradiol, or dexamethasone (**Figure 1-6**). No induction of cystatin S gene expression has been detected by RT-PCR analyses in primary cultures under different conditions of: **1**) serum concentration (from 0-10% FCS), **2**) surface where the cells were grown (plastic, collagen, laminin), **3**) time of exposure to the agonists, and **4**) varying concentration of IPR (data not shown).

Treatment of adult rats with 6-hydroxydopamine, which depletes norepinephrine stores, destroys the ability of nerves to take up exogenous norepinephrine, selectively destroys the sympathetic nerve terminals (Hausler *et al.*, 1969; Johnsson and Sachs, 1970; Tranzer, 1968), and greatly reduces DNA synthesis in submandibular glands of IPR-treated animals. A single dose of 6-hydroxydopamine given one hour prior to the administration of IPR completely eliminated the DNA synthesis induced by IPR in rat submandibular glands (Barka *et al.*, 1972). Furthermore, the increase in DNA synthesis induced by IPR *in vivo*

could not be reproduced *in vitro*; however, if rats are treated with IPR before the submandibular glands are removed and cultured *in vitro*, DNA synthesis proceeds normally (Barka, personal communication), indicating that the DNA synthesis machinery of the submandibular glands in culture remains functional. This suggests that the acinar cells require exposure *in vivo* to other sympathetic factor(s) in addition to IPR in order to be able to initiate the series of events leading successfully to initiation of DNA synthesis and replication. Again, experiments analyzing the increase in size of the rat parotid gland induced by a dietary change from all liquid to solid food, as discussed previously, provides additional support to this hypothesis. This change in diet is followed by an average increase of 200% in [^{3}H]thymidine uptake into the parotid gland. However, removal of either parasympathetic or sympathetic innervation to the parotid gland prior to the dietary change resulted in a partial inhibition of the increase and removal of both autonomic nerves resulted in complete inhibition of [^{3}H] thymidine uptake into the parotid glands (Schneyer *et al.*, 1992b). Considered together, these data suggest that at least some of the effects of β_1-adrenergic receptor stimulation *in vivo* (e. g. induction of DNA synthesis, and expression of specific set of genes) are not regulated directly via cAMP, and that additional factor(s) from sympathetic and/or parasympathetic nerve terminals are required to have the full spectrum of responses observed *in vivo* in the rat submandibular gland after treatment with IPR.

A growing number of neuropeptides has been identified that appear to play an important role in the regulation of the submandibular gland metabolism (Ekström, 1987). Vasoactive intestinal peptide (VIP) and secretin enhance norepinephrine (NE) and substance P (SP) mediated secretion of fluid and protein from rat submandibular glands (Iwabuchi and Masuhara, 1994a; Iwabuchi and Masuhara, 1994b). SP, VIP and calcitonin gene-related peptide (CGRP) are most likely involved in the non-adrenergic, non-cholinergic (NANC) secretion of saliva in response to stimulation of parasympathetic innervation (Ekström, 1987; Månsson *et al.*, 1990; Salo *et al.*, 1995). Galanin, another neurotransmitter, acts by inducing hyperpolarization of resting membrane potential in the rat submandibular gland (Konopka *et al.*, 1992). There are limited physiological data on the effects of neuropeptide Y (NPY), the most abundant sympathetic neuropeptide in the salivary glands. Local, intra-arterial infusion of NPY induces a dose-dependent vasoconstriction in cat submandibular gland (Lundberg *et al.*, 1982b), and NPY has also been proposed to be responsible for increased amylase secretion in parotid glands (Sharkey *et al.*, 1989). Based on the distribution of NPY-fibers, it has been suggested that in addition to its vascular effect, NPY may also have a functional role in the regulation of secretion from the parotid, sublingual and, possibly, the submandibular gland of the rat (Schultz, 1994).

Interestingly, the rat submandibular and parotid glands become supersensitive not only to β-adrenergic agonists but also to sympathetic neuropeptides as a result of autonomic denervation (Ekström *et al.*, 1983).

In addition to their role in synaptic communication, neurotransmitters are now recognized to play an important trophic role in cell differentiation within both the central nervous system and peripheral neural target tissues (Wagner *et al.*, 1995). Rats denervated at birth by 6-hydroxydopamine treatment showed an impaired response to vasopressin or angiotensin in stimulating cardiac ODC activity. Responsiveness of the kidney was affected only for vasopressin, and in the lung denervation had only transient effects on hormonal responses. This confirmed that sympathetic input is required for proper development of some, but not all, hormonal responses, and indicates that the role of neuronal factors is tissue specific (Hou *et al.*, 1989b).

There is increasing evidence that neuropeptides exert receptor-mediated effects on gene expression. VIP, secretin, and peptide histidine isoleucine (PHI) increased the expression of tyrosine hydroxylase (TH) gene (the rate-limiting enzyme in the catecholamine biosynthetic pathway), and this effect was mediated by the cAMP second messenger pathway (Wessels-Reiker *et al.*, 1993). VIP also stimulated c-*fos* and c-*myc* gene expression in several breast cancer cell lines (Zia *et al.*, 1996). VIP and norepinephrine induced massive glycogen synthesis in mouse cortical astrocytes that was mediated by specific receptors coupled to the

cAMP signal transduction cascade in a protein synthesis- dependent fashion (Sorg and Magistretti, 1992). More recently, it has been shown that glycogen synthesis induced by VIP, pituitary adenylate cyclase-activating peptide (PACAP) and norepinephrine was mediated by induced expression of the transcription factors CCAAT/Enhancer Binding Protein (C/EBP)-β and C/EBPδ (Cardinaux and Magistretti, 1996). There are also examples of negative regulation of gene expression by neuropeptides (Nagao *et al.*, 1995). The fact that several neuropeptides regulate the expression of different transcription factors reinforces the notion that they play an important role in the physiology of their peripheral target tissues, and suggests that they may establish complex interactions in the regulation of gene expression in those tissues.

The fact that the expression of the cystatin S gene in response to IPR in surgically sympathectomized (bilateral and unilateral) and chemically (reserpine) denervated submandibular glands is not totally suppressed but reduced to about the same extent suggests that other factor(s), in addition to the β-adrenergic pathway, participate in its regulation. The content of neuropeptides during ontogenesis may reflect not only events such as growth of neurons and organization of synapses, but also events such as functional maturation of nerve impulses, and this maturation may involve changes in the rate of synthesis and release of different sets of neuropeptides. In addition to norepinephrine, the major neurotransmitter in the sympathetic nerve terminals, other neurotransmitters or neuropeptides are also

depleted by sympathectomy, and it is possible that one or more of these factors participate in the induction of the cystatin S gene by IPR. Interestingly, the concentrations of VIP, CGRP and SP are increasing during the development of the submandibular gland when the cystatin S gene is maximally expressed (Ekström *et al.*, 1994). Furthermore, since the two branches of the autonomic nervous system act in parallel in the submandibular gland, the parasympathetic nerve terminals may also provide releasing factor(s) that are candidates for playing a role in IPR-induced expression of the cystatin S gene.

CHAPTER IV

CYSTATIN S GENE EXPRESSION INDUCED BY ISOPROTERENOL DURING EARLY DEVELOPMENT OF THE RAT SUBMANDIBULAR GLAND

INTRODUCTION

The submandibular gland of the rat begins to develop on the 13th intrauterine (i.u.) day. On i.u. days 15 and 16, cholinergic nerves make contact with the cells of the terminal buds which subsequently differentiate to form the majority of the functional structures of the gland (Cutler and Chaudhry, 1974). The first secretory material is seen on the 18th i.u. day, and by the time of birth, the gland is already actively secreting (Redman, 1987). The parasympathetic nerves are able to stimulate secretion at birth in the submandibular gland, whereas the sympathetic nerves reach the submandibular gland by postnatal day 5, and stimulate secretion beginning on postnatal day 6 (Bottaro and Cutler, 1984; Cutler *et al.*, 1981). β-adrenergic receptors are found on the surface of secretory cells as early as day 1 after birth; between days 4 and 6, the number of β-adrenergic receptors increases four-fold, concomitant with growth of nerves containing

catecholamines into the parenchyma of the submandibular glands (Bottaro and Cutler, 1984; Cutler *et al.*, 1981).

The results of the experiments presented in Chapter II show that surgical sympathectomy in adult female rats by removing the superior cervical ganglion (unilaterally or bilaterally), or chemical sympathectomy (by treatment with reserpine), reduced the expression of the cystatin S gene induced by IPR, in spite of the well documented supersensitivity of the gland to β-adrenergic agonists, and the upregulation of the β_1-adrenergic receptors in rat submandibular glands under those experimental conditions. These results strongly suggest that additional factor(s) from the sympathetic nervous system participate in the IPR-induced expression of the cystatin S gene.

Since sympathetic innervation of the rat submandibular gland is not fully developed in newborn animals, and catecholamine-containing neurons are not seen in the parenchyma of submandibular glands before day 5 of age (Bottaro and Cutler, 1984; Cutler *et al.*, 1981), such a non-innervated submandibular gland offers an excellent experimental model to test the hypothesis that an intact sympathetic innervation is required for IPR-induced expression of the cystatin S gene. This Chapter describes experiments designed to test this hypothesis by analyzing IPR-induced expression of the cystatin S gene in the rat submandibular gland during early postnatal development, before (3 and 4 days of age) and after (5, 6 and 8 days of age) the sympathetic nerve terminals reach the gland. The role

of an intact sympathetic innervation was also tested by the analysis of IPR-induced cystatin S gene expression in denervated and non-denervated submandibular glands of female rats of 4 and 8 days of age that have been unilaterally sympathectomized by removing the superior cervical ganglion on postnatal day one. Since sympathetic fibers only reach the rat submandibular gland by postnatal day 5, unilateral sympathectomy on day one allows the analyses in the same animal, of the effect of IPR on cystatin S gene expression in partially or completely innervated glands (non sympathectomized glands) and glands that have never received sympathetic innervation (sympathectomized glands).

Methods

The methods used for IPR-treatment of the animals, unilateral sympathectomy, Northern blots, quantitative and statistical analyses of the data have been described in the General Methods Chapter.

The expression of the IPR-induced expression of the cystatin S gene during early development of the rat submandibular gland was analyzed in female Sprague-Dawley (Charles Rivers) pups (10 per group, except where noted) of 3, 4, 5, 6, and 8 days of age treated with a single *i.p.* injection of IPR. The effect of sympathectomy on the IPR-induced expression of the cystatin S gene was analyzed at 4 and 8 days of age in animals that have been unilaterally sympathectomized at one day of age.

RESULTS

IPR-induced expression of the cystatin S gene during early development of the rat submandibular gland

As observed in the submandibular glands of adult female rats (Shaw *et al.*, 1990), cystatin S mRNA was not detected by Northern blot hybridization in total RNA extracted from the submandibular glands of untreated animals early in development (**Figure 4-1, A & B**). However, IPR induced cystatin S mRNA in the submandibular glands of animals at 3, 4, 5, 6 and 8 days of postnatal development.

In order to quantitate the response of the submandibular glands in developing animals to IPR, ten animals were treated with IPR at each day of postnatal development as described in the Materials and Methods section. These data indicate that submandibular glands are able to express the cystatin S gene in response to IPR during early postnatal development at 3, 4, 5, 6, and 8 days (**Figure 4-2**). It is noteworthy that there was variation in cystatin S gene expression among the animals in response to IPR, especially between 3 to 6 days of age. Northern blots demonstrated little variation in the expression of cyclophilin mRNA. Statistical analyses of these data are presented in **Figure 4-3.** The levels of IPR-induced Cystatin S mRNA in the submandibular glands of day 3, 4, 5, and 6 day old animals were not significantly different from each other. However, at day 8, IPR-induced cystatin S mRNA levels were higher, and the values were

statistically different from the levels of Cystatin S mRNA at the earlier time points (3, 4, 5, 6 days).

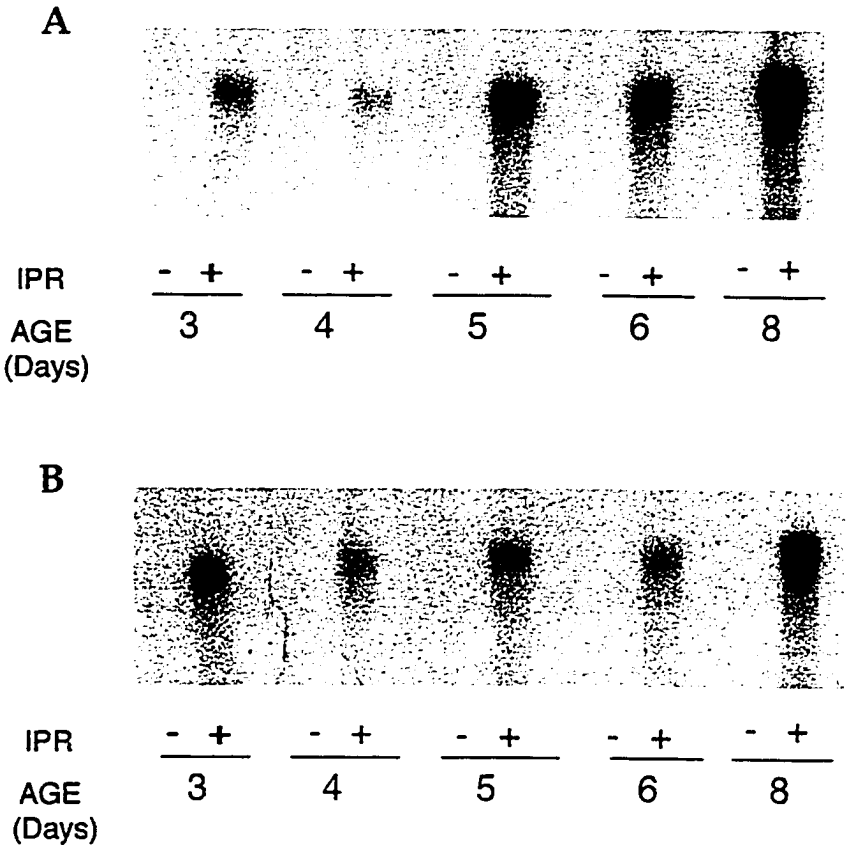


Figure 4-1. Northern blot analyses of cystatin S mRNA from submandibular glands of female rats of 3, 4, 5, 6 and 8 days of age, untreated or treated with IPR. Total RNA was extracted, hybridized and washed as described in Materials and Methods. Three micrograms of total RNA was loaded into each well. Northern blots **A & B** are from two representative experiments.

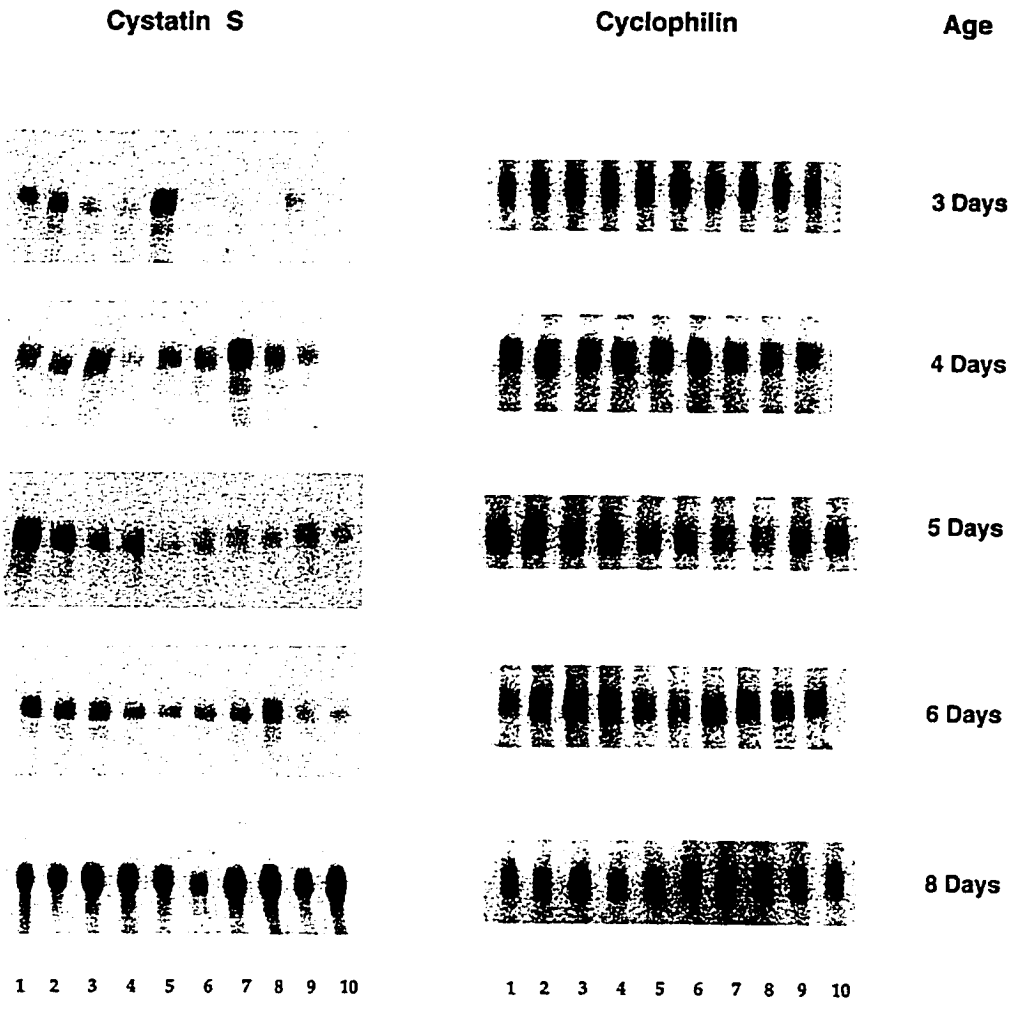


Figure 4-2. Northern blot analyses of Cystatin S mRNA from submandibular glands of female rats of 3, 4 , 5, 6, and 8 days of age treated with IPR. Three micrograms of total RNA were electrophoresed, and the Northern blots were hybridized with a cystatin S-specific riboprobe or a cyclophilin-specific riboprobe. Lanes 1-10 represent total RNA extracted from 10 animals with the exception of the 4-day old group which consisted of 9 animals.

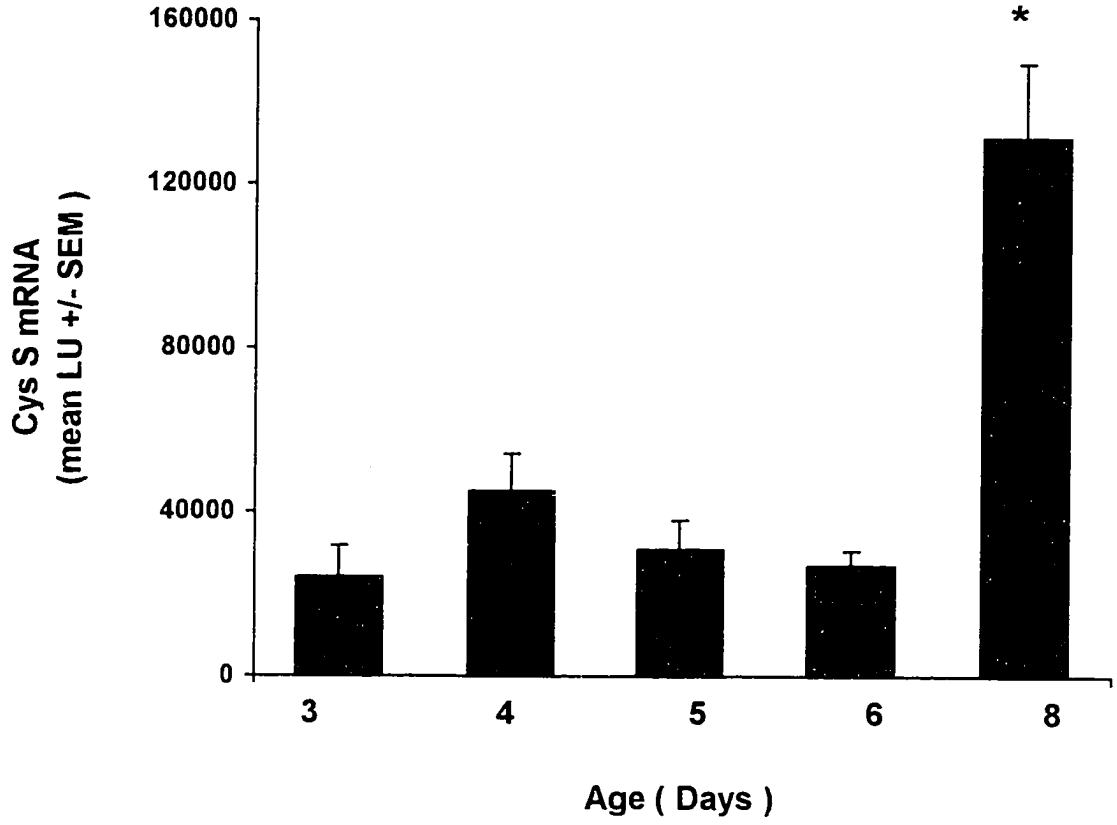


Figure 4-3. Quantitative analyses of IPR-induced cystatin S gene expression during early development of the rat submandibular gland. Northern blots in Figure 4-2 were quantitated using the PhosphorImager as described in General Methods. The values are expressed as cystatin S mRNA light units (Mean LU +/- SEM) and the data were analyzed by one way ANOVA and the *post-hoc* analysis was performed by the Tukey-HSD multiple range test with the significance level set at 0.05. Each data point represents the mean of 10 (9 for day 4) values.

(*) Differences with other groups are statistically significant.

The submandibular glands from untreated animals at 3, 4, 5, 6, and 8 days of development were microscopically examined using PAS staining, and **Figure 4-4** shows representative photomicrographs. The morphological examination of these sections indicated a progressive differentiation of the glands during the period of time examined, showing a decrease in the number proacinar cells (pyramidal shaped cells containing PAS-positive granules and showing an intense red staining) and an increase in the number of acinar cells (pyramidal shaped cells, with a paler PAS staining, as result of changes in the structure and composition of secretory granules). By day 5, virtually no proacinar cells were observed and in most of the acinar cells secretory granules began to accumulate in the cytoplasm. At day 8, the cytoplasm of acinar cells was filled with secretory granules. **Figure 4-5** illustrates H & E stained sections of control versus IPR-treated submandibular glands at the same developmental time points as seen in Figure 4. On day 3, IPR had little effect on the morphology of the acinar cells (**Figure 4-5 A & B**). By day 5, secretory granules in the acinar cells are beginning to accumulate in response to IPR (**Figure 4-5 C & D**), and by day 8, there was a large accumulation of secretory granules in the acinar cells, resembling those observed in adult glands (**Figure 4-5 E & F**).

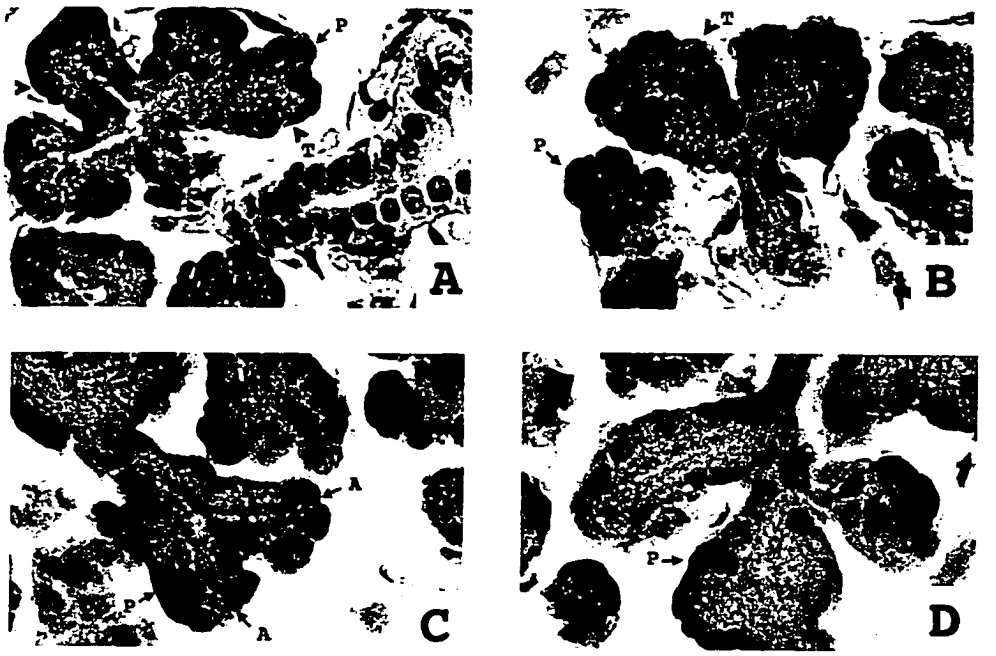


Figure 4-4. Submandibular glands of developing female rats, fixed in paraformaldehyde, embedded in paraffin, and stained by the PAS reaction. X250. **A and B.** submandibular glands from 3 day old animals. Developing acini containing proacinar cells (P), terminal tubule cells (T), and acinar cells in different stages of differentiation (small arrows). **C.** submandibular gland from a 5 day old animal. Note the decrease in the number of the proacinar cells (P), and the increase in the number of developing acinar cells (A), characterized by the accumulation of secretory granules. **D.** submandibular gland from a 8 day old animal. Most of the cells in the acini are fully differentiated acinar cells with very few proacinar (P) cells remaining.

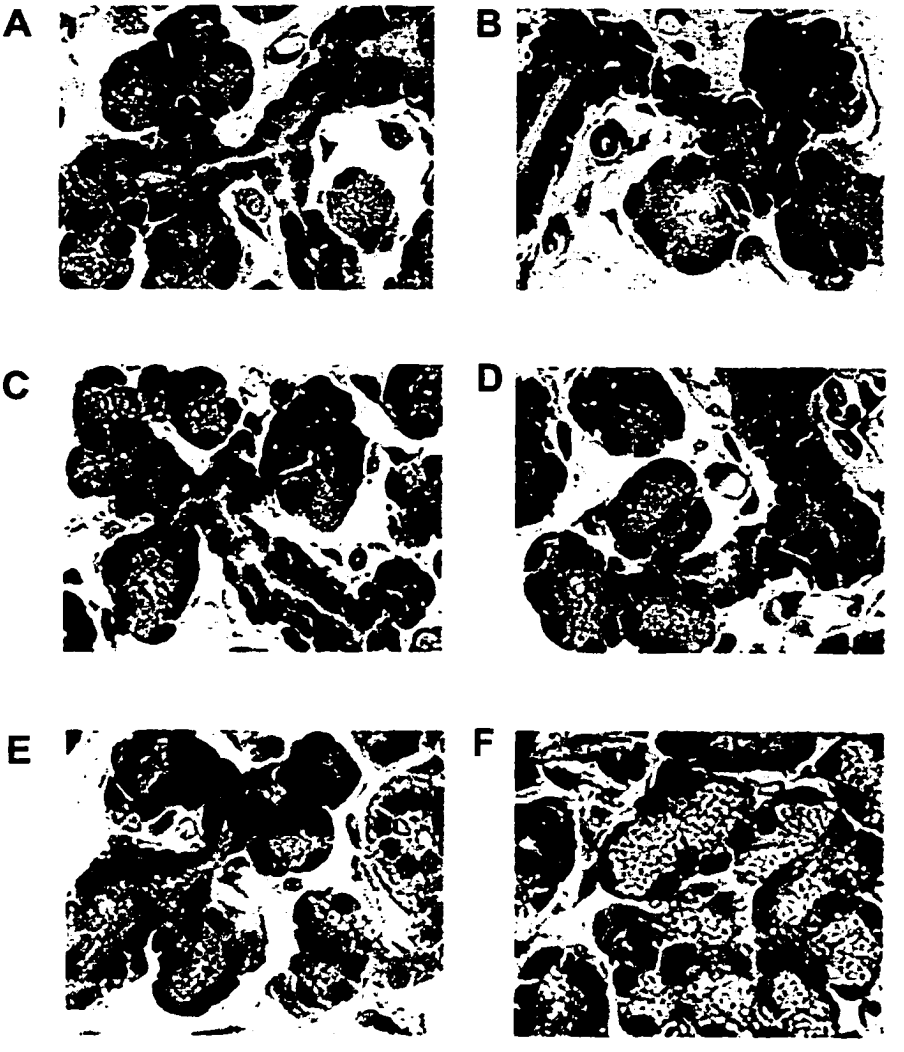


Figure 4-5. Submandibular glands of developing female rats untreated and treated with IPR, fixed in paraformaldehyde, embedded in paraffin, and stained with H & E. X250.
A. submandibular gland from a 3 day old control animal, **B.** submandibular gland from a 3 day old IPR-treated animal, **C.** submandibular gland from a 5 day old control animal, **D.** submandibular gland from a 5 day old IPR-treated animal, **E.** submandibular gland from a 8 day old control animal, **F.** submandibular gland from a 8 day old IPR-treated animal. In submandibular glands of 3 and 5 day old rats, IPR did not induce clear morphological changes. At 8 days of age the IPR-treated gland showed a very conspicuous enlargement of the acini and accumulation of secretory material reminiscent of the response of the adult gland.

Effect of unilateral sympathectomy

Next, the effect of sympathectomy on IPR-induced expression of the cystatin S gene in submandibular glands at 4 and 8 days of development was examined in rats that had been unilaterally sympathectomized at one day of age and comparing the expression of cystatin S gene in the control, non-denervated gland with that in the sympathectomized gland. Northern blot analyses demonstrated that at both 4 and 8 days there was a reduction of IPR-induced cystatin S mRNA in sympathectomized submandibular glands (**Figure 4-6 A & B**). Statistical analyses by Student's *t*-test for paired samples of the Northern blots indicated that the differences in cystatin S mRNA induced by IPR in sympathectomized submandibular glands compared to those of the control glands was significant at both time points analyzed, with p values of 0.048 at 4 days, and 0.023 at 8 days (**Figure 4-7**).

Control and sympathectomized submandibular glands from rats treated with IPR at 4 and 8 days of age were examined microscopically in sections stained by the PAS staining (**Figure 4-8**) and sections stained with H & E (**Figure 4-9**). Sympathectomy caused no conspicuous alterations in the submandibular glands and no obvious changes in the frequency of proacinar or acinar cells were seen when control, non sympathectomized glands and sympathectomized glands were compared.

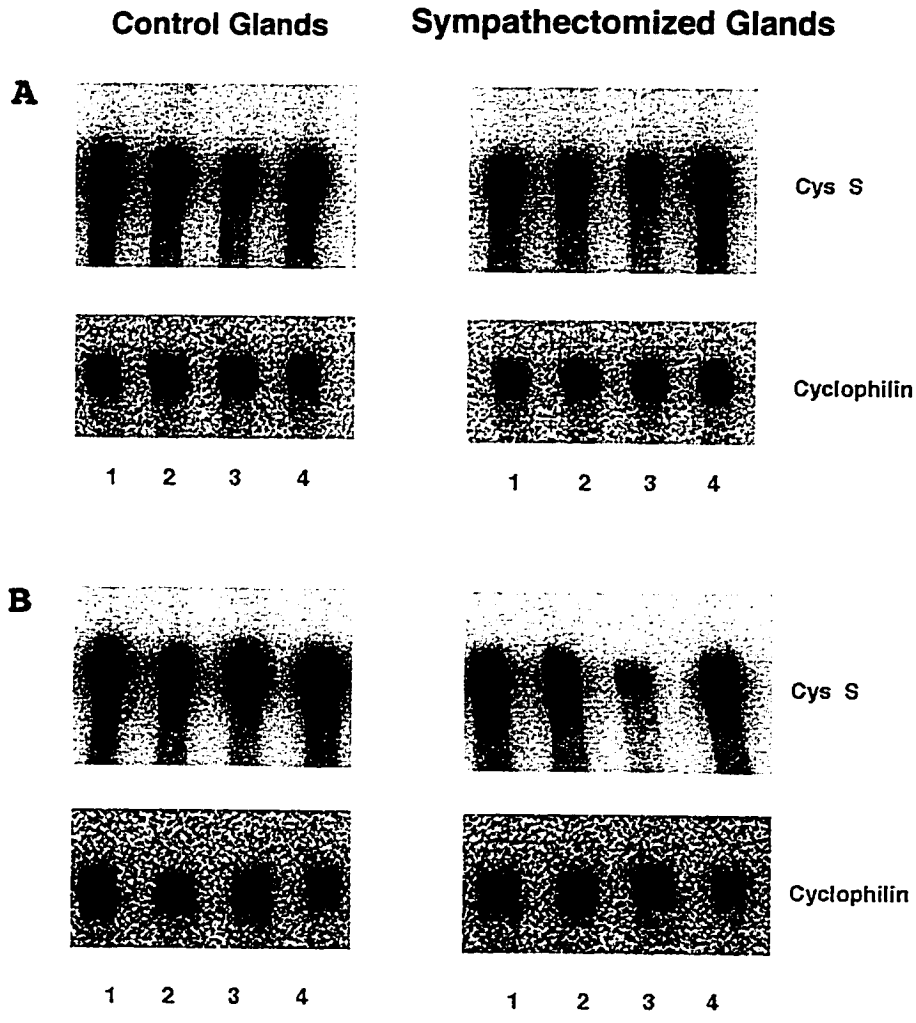


Figure 4-6. Northern blot analyses of cystatin S mRNA in submandibular glands of unilaterally sympathectomized, IPR-treated female rats at 4 and 8 days of development. Three μg of total RNA were electrophoresed, and the Northern blots were hybridized with a ^{32}P-labeled cystatin S-specific riboprobe or a ^{32}P-labeled cyclophilin specific riboprobe as described in Materials and Methods. **A.** Northern blot analyses from 4 unilaterally sympathectomized 4-day old animals (lanes 1-4). Left gland, intact gland; right gland, sympathectomized gland. **B.** Northern blot analyses from 4 unilaterally sympathectomized 8-day old animals (lanes 1-4).

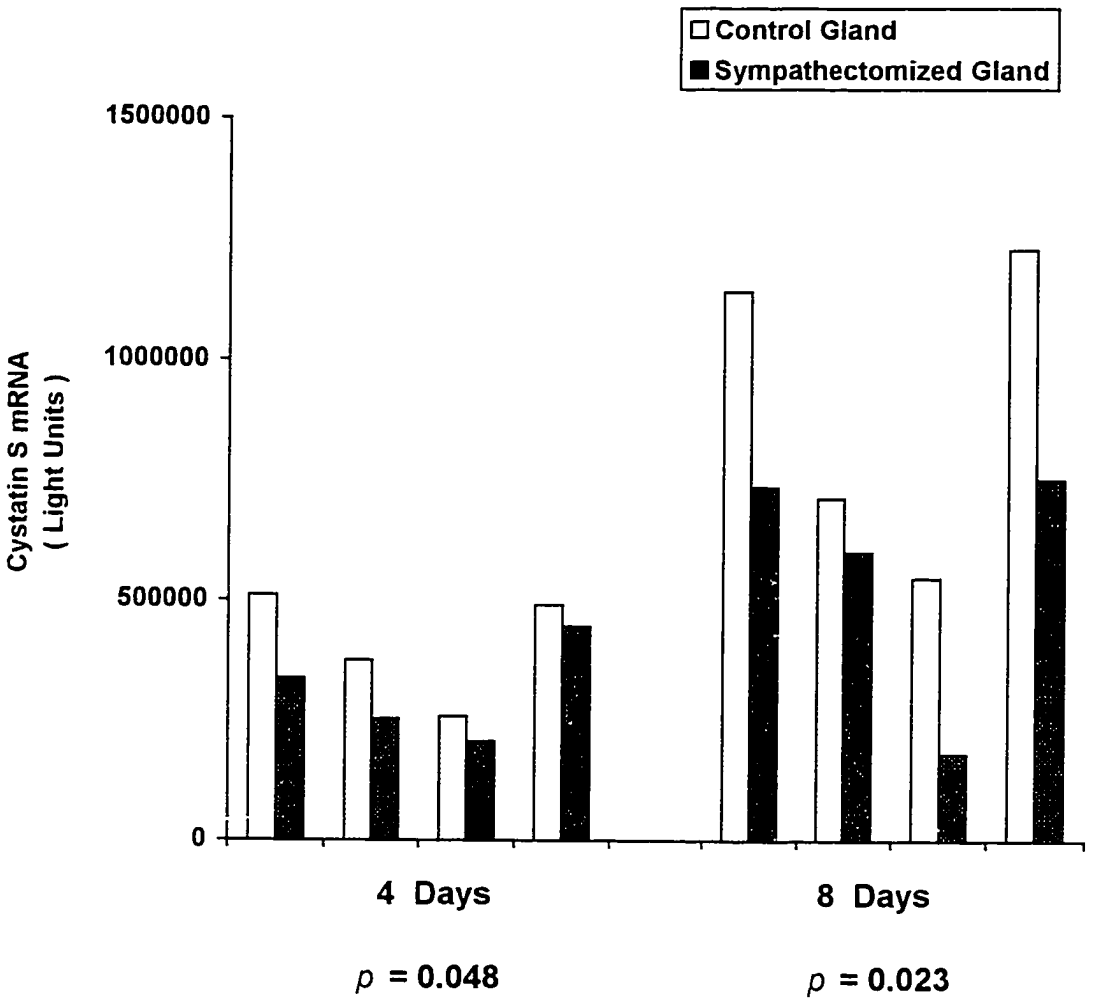


Figure 4-7. Effect of unilateral sympathectomy on the IPR-induced expression of the cystatin S gene during early development of the rat submandibular gland. Quantitative analyses of the Northern blots in Figure 4-6 were performed using the PhosphorImager (Molecular Dynamics). The data are expressed in cystatin S mRNA light units and were analyzed by a Student's *t*-test for paired samples. Each pair of bars represent the control and sympathectomized gland from the same animal.

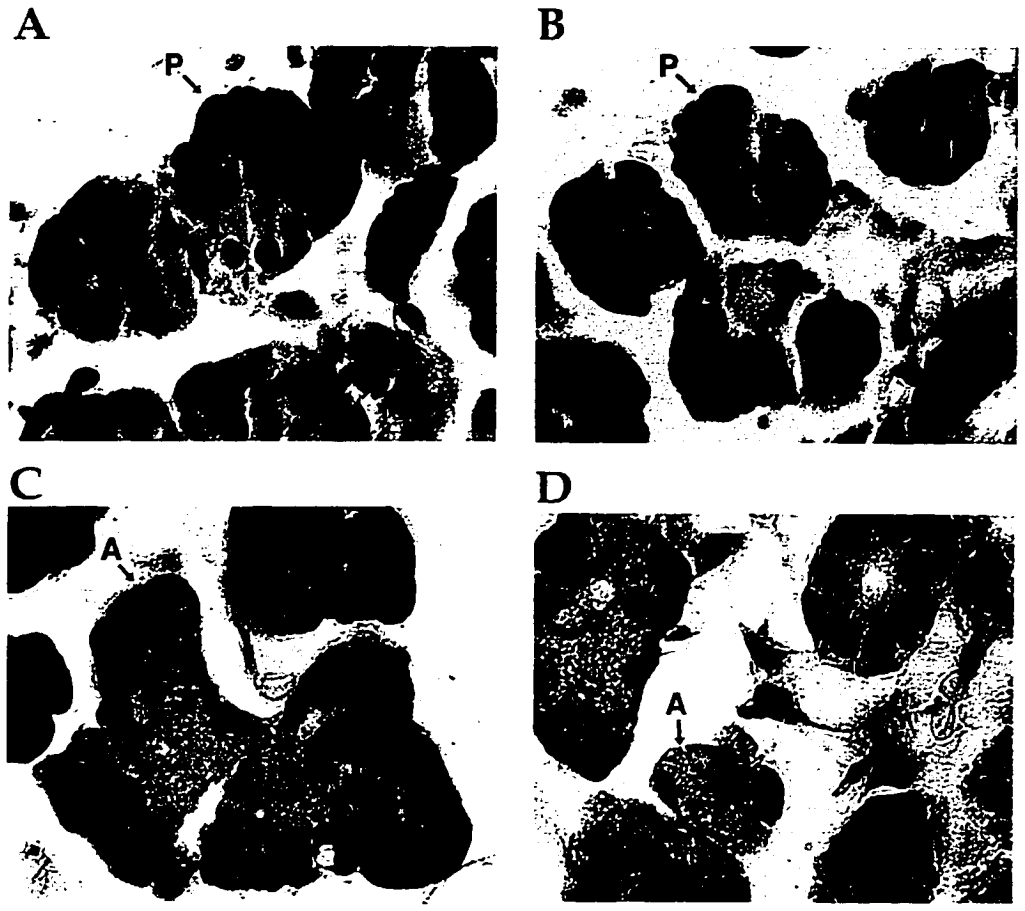


Figure 4-8. Submandibular glands of developing female rats sympathectomized unilaterally at one day of age and treated with IPR, fixed in paraformaldehyde, embedded in paraffin, and stained by the PAS reaction. X250. **A.** Control, non-denervated and **B.** denervated submandibular gland from an unilaterally sympathectomized animal treated with IPR at 4 days of age. **C.** Control, non-denervated and **D.** denervated submandibular gland from an unilaterally sympathectomized animal treated with IPR at 8 days of age. Developing acini containing proacinar cells (**P**), (**A**) acinar cells, and proacinar cells in different stages of differentiation. No morphological changes between control and sympathectomized glands were observed at these time points during early development.

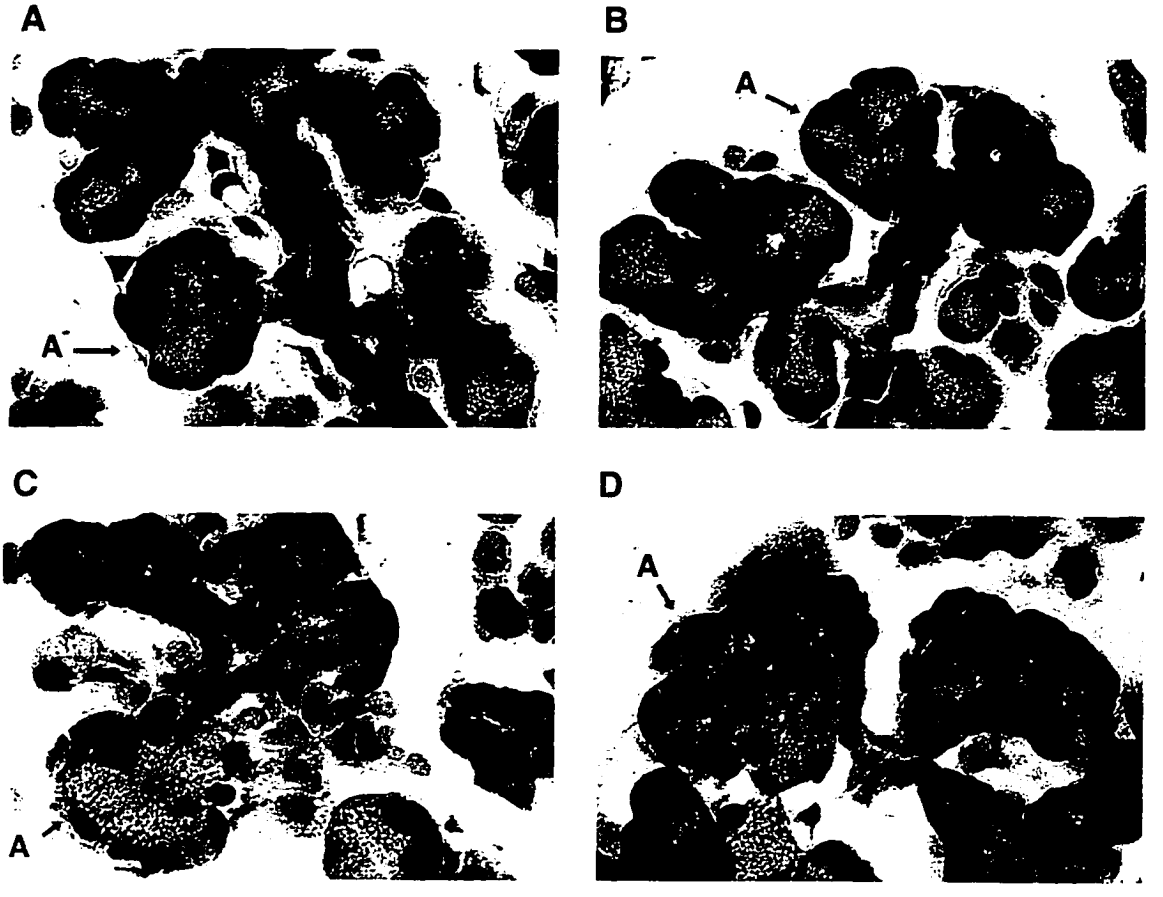


Figure 4-9. Submandibular glands of developing female rats sympathectomized unilaterally at one day of age and treated with IPR, fixed in paraformaldehyde, embedded in paraffin, and stained with H & E. X250. **A.** Control, non-denervated submandibular gland and **B.** sympathectomized gland from an unilaterally sympathectomized animal treated with IPR at 4 days of age. **C.** Control, non-denervated and **D.** sympathectomized gland of an unilaterally sympathectomized animal treated with IPR at 8 days of age. No morphological differences were observed between control and denervated glands at these time points during early development.

Discussion

In the rat submandibular gland the parasympathetic nerves are able to activate secretion at birth, whereas the sympathetic nerves reach the gland by postnatal day 5, and activate secretion beginning on postnatal day 6 (Bottaro and Cutler, 1984; Cutler *et al.*, 1981). β-adrenergic receptors are found on the surface of secretory cells as early as day 1 after birth (Bylund *et al.*, 1982), and they are functional since they are able to induce peroxidase secretion in response to IPR stimulation (Cutler *et al.*, 1981; Yamashina and Barka, 1972). The results of the experiments described in this Chapter are consistent with previous reports indicating that β-adrenergic receptors present early in the developing rat submandibular gland are functional and show that they are capable of responding to IPR stimulation by inducing the expression of the cystatin S gene even at 3 and 4 days of age, when sympathetic nerve fibers have not reached the gland (**Figure 4-1 A & B, and Figure 4-2**). This indicates that sympathetic innervation is not a requisite for IPR-induced expression of cystatin S gene. However, once the submandibular gland is innervated by sympathetic nerve terminals it shows a more robust response to β-receptor stimulation and the level of expression of cystatin S mRNA induced by IPR on postnatal day 8 is significantly higher than those reached during previous days (3, 4, 5, and 6 days) (**Figures 4-2 & 4-3**). The simplest explanation for the increased expression of the cystatin S gene in

response to IPR at 8 days is that it is due to the differentiation of the submandibular gland. The analysis of several characteristic features of the developing submandibular gland during this period of time suggested, however, that this is not a complete explanation. First, the percentages of the various cell types during the postnatal development of the submandibular gland change continuously (Chang, 1973; Chang and Barka, 1973); the number of acinar cells between 2 and 29 days of age increases and the number of proacinar cells during the first week of postnatal development decreases. By 2 days of age, the acinar cells represent 1.6 % of the total number of cells in the developing submandibular gland, and this percentage increases to 16.1 % by day 7. In contrast, the number of proacinar cells decreases from 23.6 % at day 2, to 3.8 % at day 7 (Chang, 1973; Chang and Barka, 1973). The morphological analyses of the developing rat submandibular gland shown in **Figure 4-4** illustrate the continuous change in the composition of acinar and proacinar cell populations at 3, 5, and 8 days of age. The levels of cystatin mRNA in the submandibular glands of IPR-treated rats do not parallel the changes in acinar and proacinar cell populations and are not significantly different between 3 and 6 days of age (**Figures 4-2 & 4-3**), indicating that IPR-induced expression of the rat cystatin S gene during early development of the submandibular gland is not just a function of the degree of differentiation of the acinar cells. These results also suggest that proacinar cells are able to express cystatin S gene in response to IPR stimulation. Future experiments by

immunocytochemistry and/or *in situ* hybridization are required to confirm this suggestion.

Second, as discussed previously, functional β-adrenergic receptors are found in the membrane of secretory cells as early as 1 day after birth (Cutler *et al.*, 1981; Yamashina and Barka, 1972); between days 4 and 6 after birth, the number of β-adrenergic receptors increases four-fold in the rat submandibular gland, and this increase coincides with the appearance of adult-type stimulus-secretion coupling in the gland (Cutler *et al.*, 1981). This increase in the number of β-adrenergic receptors was not paralleled by a similar increase in the level of expression of the cystatin S gene induced by IPR (**Figures 4-2 & 4-4**). As in the sympathectomized adult submandibular gland, an increase in the number of β-adrenergic receptors was not accompanied by an increase in the expression of the cystatin S gene upon IPR treatment.

Third, as discussed in Chapter I, β-adrenergic receptors are coupled to the trimeric G protein-adenylate cyclase-cAMP-PKA cell signaling pathway (Kobilka, 1992). If the increase in the number of β-adrenergic receptors does not produce a comparable increase in the expression of cystatin S gene expression in response to IPR during early development of the gland, it can be argued that because of the lack of full maturation of the gland, one or more of the components of the pathway downstream from the β-adrenergic receptors may not be coupled properly to β-

adrenergic receptors. However, membrane-associated adenylate cyclase activity from the submandibular glands of 1- and 4-day old rats is stimulated reproducibly 25-40 % by IPR, and membranes from glands of 6-day old animals show activation of adenylate cyclase by IPR, similar to the 2.5 to 3.5-fold activation seen in the membranes of glands of adult rats (Cutler *et al.*, 1981). This indicates that by day 6 of postnatal development of the gland, all the components of the β-adrenergic receptor signaling pathway are functionally coupled. Again, this increase in adenylate cyclase activation by IPR was not paralleled by a corresponding increase in the level of expression of cystatin S gene in the submandibular gland of 6-day old rats (**Figures 4-2 & 4-3**).

In summary, the development of submandibular glands during the first postnatal week is a continuous process that is not reflected in a similar increase in the expression of the cystatin S gene induced by stimulation of the β-adrenergic receptors by an agonist. It was only after 6 days of age that a significant increase in cystatin S gene expression was observed in response to IPR, indicating that this expression is not entirely dependent on the β-receptor-cAMP pathway and that additional factor(s), probably from the sympathetic nervous system, are required for the full response of the cystatin S gene to IPR. The results of the unilateral sympathectomy experiments are consistent with this interpretation.

Postsynaptic adrenergic reactivity to neuronal stimuli in adult animals leads first to uncoupling of receptors from response elements to produce desensitization,

and with continued overstimulation, the number of receptors declines either because of a decrease in gene expression or because of an increased receptor turnover (Maisel *et al.*, 1987; Pointon and Banerjee, 1979; Yamada *et al.*, 1980; Yamada *et al.*, 1986). Denervation or pharmacological blockage of adrenergic receptors leads to supersensitivity and upregulation of receptor number (Ekström, 1979; Ekström, 1980; Perec *et al.*, 1975; Perec *et al.*, 1973; Stefano and Perec, 1981). In neonatal rats, however, the supersensitivity of the response to β-receptor stimulation after sympathectomy is not a general feature. Chemical sympathectomy by treatment with 6-hydroxydopamine fails to induce cardiac or hepatic upregulation of α_1- or β-adrenergic receptors, and supersensitivity was completely absent (liver) or emerged only transiently several weeks after 6-hydroxydopamine treatment (heart) (Slotkin *et al.*, 1995). Interestingly, the ability of IPR to stimulate ornithine decarboxylase (ODC), a growth related enzyme, in heart, lung and kidney is reduced by neonatal sympathectomy (Hou *et al.*, 1989a), and it has been observed that lesioning noradrenergic nerves of one day old rats with 6-hydroxydopamine produces a loss of the ability of the β-adrenergic receptors to induce *c-fos* expression, resulting in reduced expression of ODC in the rat cerebellum (Wagner *et al.*, 1995).

In submandibular glands of neonatal rats, chemical sympathectomy does not affect the time of appearance or the number of β-adrenergic receptors (Cutler *et al.*, 1985), indicating that the rat submandibular gland does not become

supersensitive after chemical sympathectomy during early development. However, when unilaterally sympathectomized newborn rats are chronically treated with IPR between 22 and 26 days of age, the non-innervated glands consistently show greater absolute and relative weight, and total DNA, RNA and protein than the corresponding intact gland from the same animal (Gresik and Barka, 1977), resembling the delay of appearance of supersensitivity observed in the heart (Slotkin *et al.*, 1995).

The results of the sympathectomy experiments presented in this Chapter confirm that an intact sympathetic innervation of the submandibular gland is not absolutely required for the expression of the cystatin S gene induced by IPR. However, as indicated by the comparison of the levels of cystatin S mRNA induced by IPR in the innervated and non-innervated glands of 8-day old animals (**Figures 4-6 & 4-7**), sympathetic factor(s) are required for the full IPR-induction of the cystatin S gene expression. Moreover, these results are similar to those of the sympathetic denervation experiments in adult rats (Chapter III), and show that the reduction in the level of cystatin S gene expression induced by IPR is the result of depriving the denervated gland of sympathetic factor(s), independently of the condition of β-adrenergic agonist supersensitivity in the submandibular gland after sympathectomy, i.e, no supersensitivity in the newborns or supersensitivity in adults. The reduction of IPR-induced level of cystatin S mRNA in the denervated gland of 4-day old animals as compared to the innervated gland perhaps reflects

the fact that differentiation is a continuous process and not an all or none phenomenon.

The neurotransmitter and neuropeptide phenotype expressed by the precursor neurons and the mature neurons they give rise to can change during the course of development; however, the mechanisms that control this expression are not well understood (Habecker *et al.*, 1996). The fact that a significant increase in the level of cystatin S gene expression induced by IPR in the developing submandibular gland is not observed before day 8 of postnatal development and not by the time the sympathetic nerve terminals reach the gland (postnatal day 5), may reflect a maturation process at the synapses. The observation that sympathetic neurons alter their expression of neuropeptides during development indicates that these neurons are plastic with regard to neurochemical properties, and raises the possibility that extracellular signals contribute to alterations in phenotype. A number of investigators have noted that the transmitter repertoire expressed by mature neurons is often correlated with their particular peripheral target organ (Landis, 1990; Teitelman *et al.*, 1979), and suggested that target tissues instruct the innervating neurons to synthesize the specific neurotransmitters and neuropeptides appropriate for the target, or suppress the synthesis of unnecessary neurotransmitters and neuropeptides (Habecker *et al.*, 1996). These changes are also reflected in the changes of neuropeptide content of the developing submandibular gland (Ekström *et al.*, 1994; Virta *et al.*, 1994). In addition to

norepinephrine, the major neurotransmitter in the sympathetic nerve terminals, other neurotransmitters and neuropeptides are depleted from the submandibular gland after sympathectomy, and it is possible that one or more of them participate in IPR-induction of the cystatin S gene.

CHAPTER V

CYSTATIN S GENE EXPRESSION INDUCED BY ISOPROTERENOL IN THE SUBMANDIBULAR GLANDS OF PARASYMPATHECTOMIZED RATS

INTRODUCTION

The autonomic nervous system regulates the function of the salivary glands (Emmelin, 1964), and the amount, rate of secretion, and composition of saliva are modified by both the sympathetic ($alpha_1$-, $alpha_2$-, and $beta_1$-adrenergic) and parasympathetic (muscarinic cholinergic) receptor systems. Parasympathetic electrical stimulation induces relatively large volumes of saliva low in protein concentration, without morphological evidence of acinar or duct cell degranulation (Garret *et al.*, 1991; Martinez *et al.*, 1975).

There is increasing evidence that the regulatory role of the parasympathetic innervation of the submandibular gland is not restricted to secretory mechanisms and composition of saliva. Parasympathetic denervation (sectioning the chorda tympani nerve) in rat and mouse produces a decrease of about 30 % in the size of the submandibular glands (Katsukawa *et al.*, 1990; Peronace *et al.*, 1964). Consistent with these observations, it has also been demonstrated that electrical

stimulation of parasympathetic nerves causes a mitogenic response in the parotid and submandibular glands and an increase in the activity of the enzyme β1,4-galactosyltransferase, known to be implicated in hyperplastic responses (Schneyer *et al.*, 1993). Further evidence for a regulatory role of the parasympathetic innervation in the hyperplastic response is provided in experiments showing that the increase in size of parotid glands of rats induced after a dietary change (from all liquid to solid food), and the parallel increase in the activity of the enzyme β-1,4-galactosyltransferase, are prevented by removal of the parasympathetic innervation before the dietary change (Humphreys-Beher and Schneyer, 1987; Schneyer *et al.*, 1992b).

Surgical sympathectomy (bilateral or unilateral) and chemical sympathetic denervation (by treatment with reserpine) reduce the expression of the cystatin S gene in the submandibular glands of IPR-treated adult rats, indicating that sympathetic autonomic factor(s), not just norepinephrine, participate in its regulation (Chapter IV). Additional evidence for the requirement of sympathetic factor(s) in this regulation is provided by the analysis of the expression of the cystatin S gene in response to IPR during early development of the submandibular gland (Chapter V). The fact that the IPR-induced expression of the cystatin S gene in sympathectomized submandibular glands is not totally suppressed but reduced to about the same extent suggests that other factor(s), perhaps form the parasympathetic nervous system, may also participate in its regulation.

There is another line of evidence suggesting that neural inputs, different from the sympathetic innervation may play a role in the regulation of the cystatin S gene expression. Treatment of adult rats with papain (a cysteine protease), administered into the oral cavity twice daily for 5 days, produces the enlargement of the submandibular glands and a dramatic increase in the level of cystatin S (Naito *et al.*, 1992), and this induction of cystatin S gene expression is inhibited by glossopharyngeal denervation (Ninomiya *et al.*, 1994). This is a very interesting observation since the glossopharyngeal nerve, a motor and sensory nerve, does not innervate the submandibular gland. Postganglionic fibers of the parasympathetic nucleus innervate the parotid glands and sensations of taste from the posterior one-third of the tongue and from the pharynx travel through peripheral axons to the sensory nucleus via the glossopharyngeal nerve (Snell, 1992). Studies in several species of mammals have shown that bitter-tasting substances, such as quinine, phenylthiourea, and sucrose octa-acetate, elicit greater stimulation of the glossopharyngeal nerve than of the chorda tympani nerve, while the reverse is true for salty (e.g., NaCl) and sweet-tasting (e.g., sugars) substances. It is generally believed that the glossopharyngeal nerve is a dominant taste input for behaviorally aversive substances, while the chorda tympani nerve is predominant for acceptable substances (Hanamori *et al.*, 1988; Ninomiya and Funakoshi, 1989; Ninomiya *et al.*, 1993). The inhibition of papain-induced cystatin S gene expression by glossopharyngeal denervation suggests that chemosensory information transferred

by the glossopharyngeal nerve plays an important role in the recognition of nutrient or toxic substances in the diet, and may induce biological responses for adjustments against nutritional deficiencies and defense against exogenous toxic substances (Ninomiya *et al.*, 1994).

This Chapter describes experiments designed to test the hypothesis that neuronal factor(s) from the parasympathetic nervous system participate in IPR-induced cystatin S gene expression in rat submandibular glands.

Methods

The methods used for surgical parasympathectomy and glossopharyngeal unilateral denervation, IPR treatment of the animals, Northern blots, quantitative and statistical analyses of the data were described in the General Methods Chapter.

Due to the difficulty of removing completely the rat submandibular ganglion, surgical parasympathectomy was performed by severing bilaterally or unilaterally the chorda tympani/lingual nerves (CT/LN). The effect of bilateral parasympathectomy on the IPR-induced expression of the cystatin S gene was analyzed in submandibular glands of female adult rats, treated with IPR 3 days and 14 days after surgery. Based on the initial results of bilateral parasympathectomy experiments, quantitative analyses of the effect of surgical bilateral or unilateral parasympathectomy on IPR-induced cystatin S gene expression were performed in adult female rats 14 days after surgery. The effect of glossopharyngeal denervation

on IPR-induced expression of the cystatin S gene was analyzed at 14 days after surgery in unilaterally denervated adult female rats.

Results

Effect of bilateral parasympathectomy on IPR-induced expression of the cystatin S gene in rat submandibular glands

Bilateral parasympathectomy by severing the CT/LN in adult rats reduced the IPR-induced expression of the cystatin S gene, compared to the level of expression in sham operated animals. **Figure 5-1 A**, depicts the results of Northern blot analyses indicating that the level of cystatin S mRNA was reduced as early as three days after bilateral parasympathectomy of the submandibular gland. The levels of cystatin S mRNA are even more reduced in the submandibular glands of rats treated with IPR 14 days after the CT/LN have been severed, compared to those in sham operated animals, treated similarly with IPR (**Figure 5-1 A, B & C**). A more extensive analysis was performed in a group of five sham-operated controls and five bilaterally parasympatectomized animals treated with IPR 14 days after surgical parasympathectomy (**Figure 5-2**). Quantitative analyses of the Northern blots indicate that the level of cystatin S gene expression in bilaterally parasympathectomized submandibular glands of IPR treated rats is reduced to approximately 32.6 % of that of the sham-operated animals (**Figure 5-3,** p=0.027, n=5). This effect of bilateral parasympathectomy on IPR-induced expression of the

cystatin S gene in rat submandibular glands resembles the effect of bilateral sympathectomy (see Chapter III, **Figures 3-3, 3-4 & 3-5**). It should also be noted that, as in the case of the bilateral sympathectomy, removing the CT/LN had no effect by itself on cystatin S gene expression in the absence of IPR treatment (**Figure 5-1**).

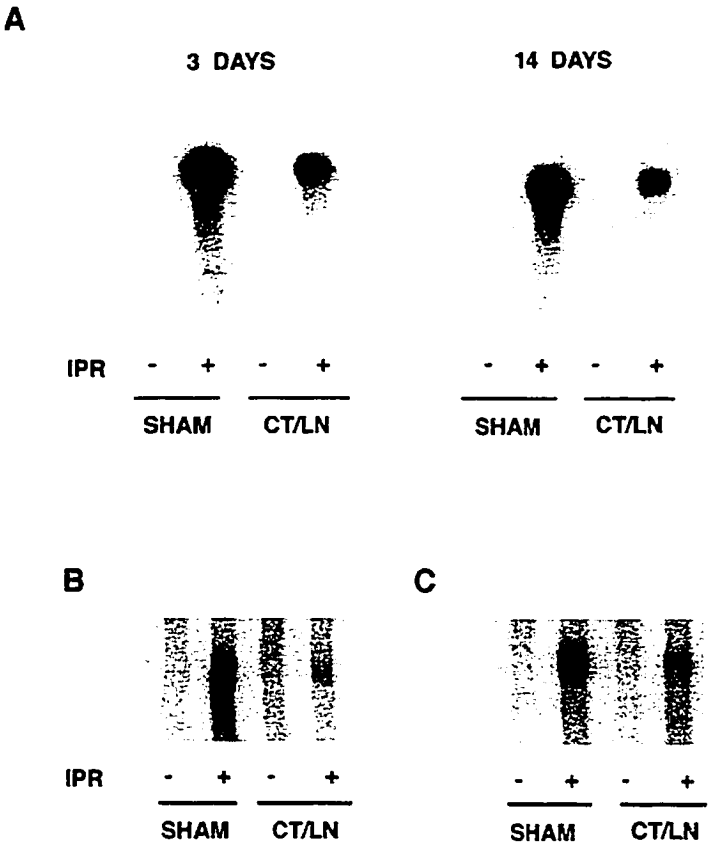


Figure 5-1. Northern blot analyses of cystatin S mRNA from submandibular glands of shams and bilaterally parasympathectomized adult female rats untreated or treated with IPR, 3 or 14 days post denervation. A. Northern blot analyses 3 and 14 days after surgery. **B and C.** Two different experiments fourteen days after surgery.

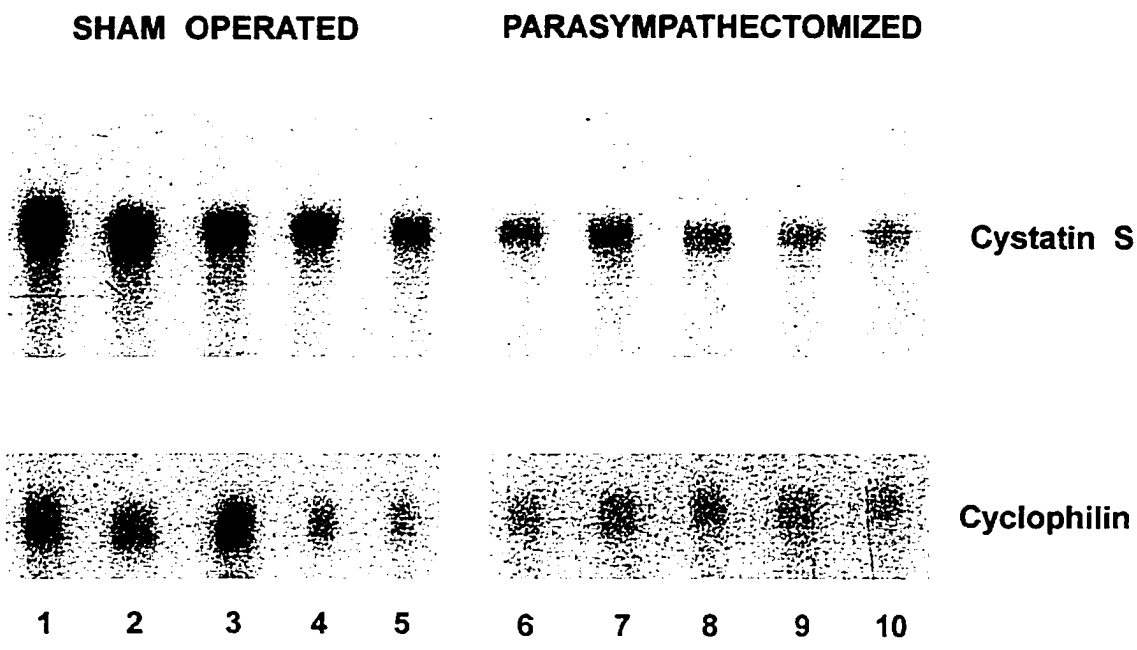


Figure 5-2. Northern blot analyses of cystatin S mRNA from submandibular glands of sham-operated and bilaterally parasympathectomized adult female rats treated with IPR 14 days after surgery. Lanes 1-5, five sham-operated, adult female rats treated with IPR. Lanes 6-10, five bilaterally parasympathectomized adult female rats treated with IPR.

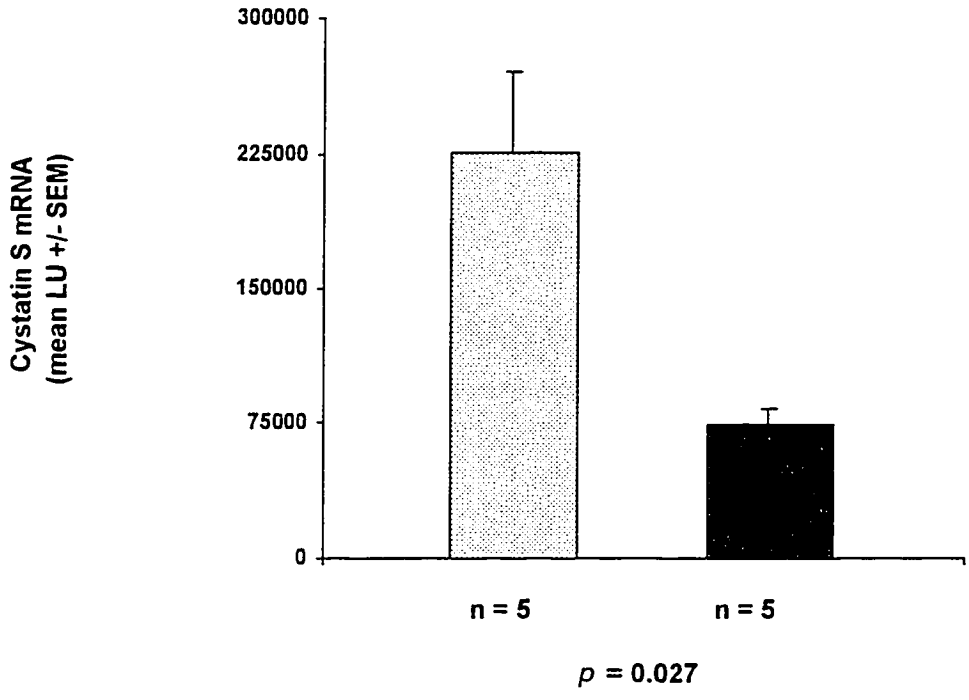


Figure 5-3. Cystatin S mRNA expression in submandibular glands of sham and bilaterally parasympathectomized, IPR-treated rats. Quantitative analyses were performed using the PhosphorImager (MolecularDynamics). The data are expressed as Light Units (Mean LU +/- SEM) and analyzed by a *t*-test for independent samples. Quantitative analyses are from the Northern blots represented in Figure 5-2.

Effect of unilateral parasympathectomy on the IPR-induced expression of the cystatin S gene in rat submandibular glands

Surprisingly, the results of unilateral parasympathectomy experiments showed that the levels of cystatin S mRNA induced by IPR in the denervated glands were not significantly different when compared with the levels of cystatin S mRNA in the non-denervated glands in the same animal, as analyzed by the *t*-test for paired samples ($p = 0.581$; n = 5), (**Figures 5-4 & 5-5**).

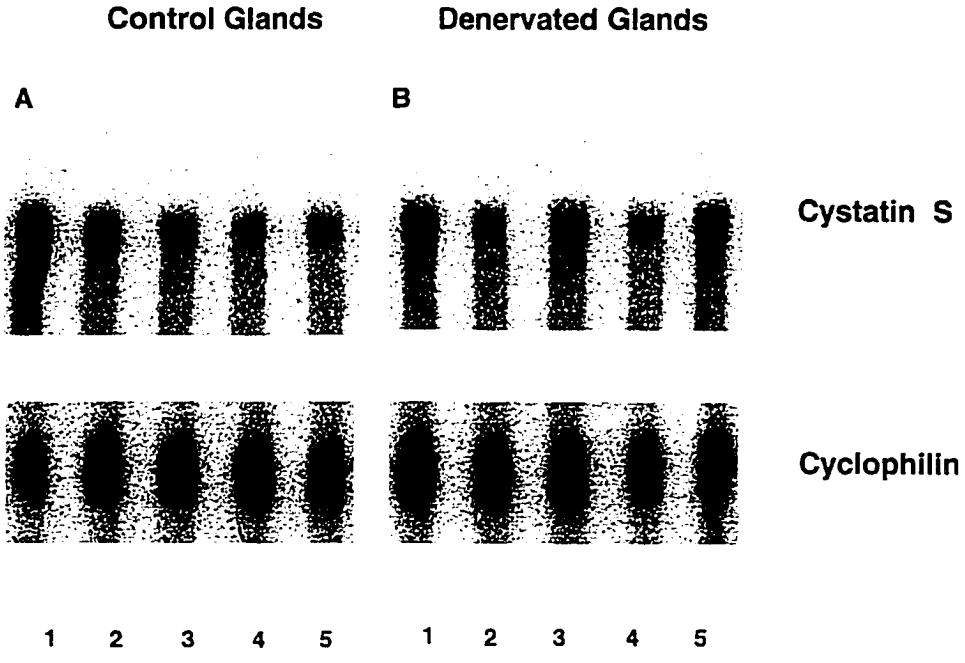


Figure 5-4. Northern blot analyses of cystatin S mRNA from submandibular glands of unilaterally parasympathectomized adult female rats treated with IPR. A. Total RNA from left glands (intact, non-denervated glands). **B.** Total RNA from right, parasympathectomized submandibular glands.

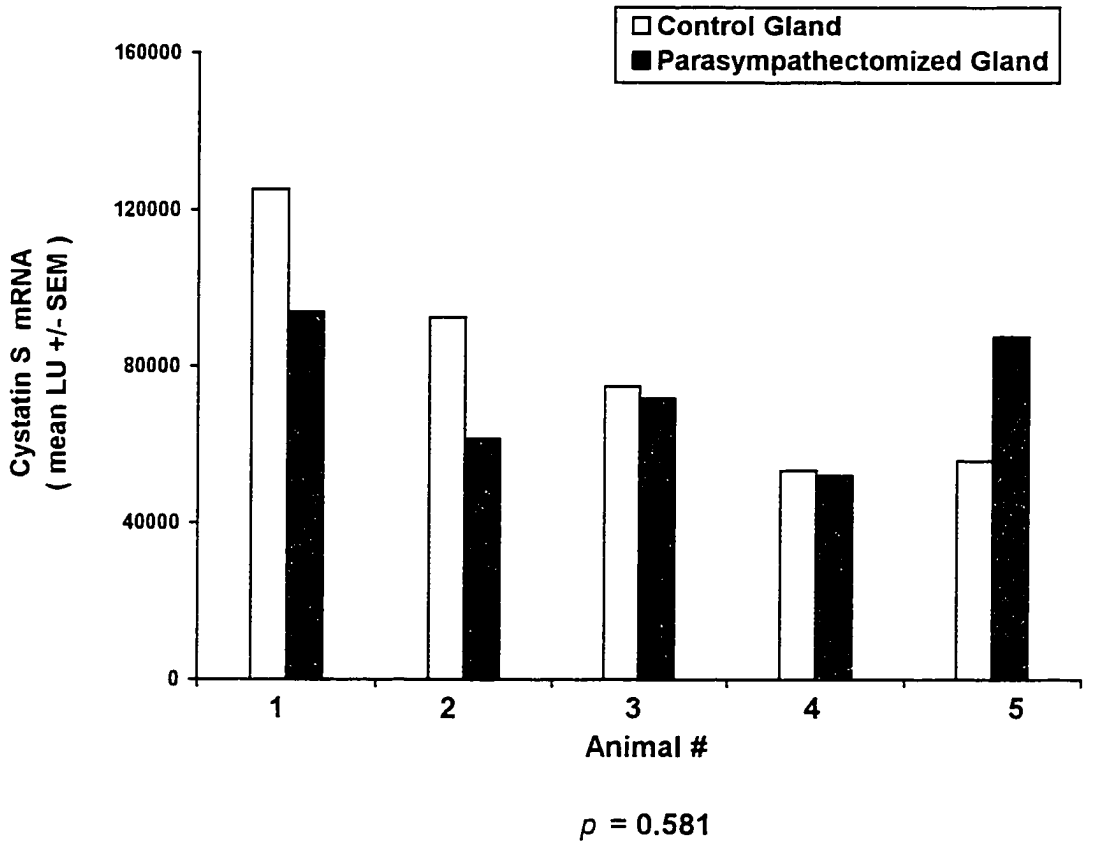


Figure 5-5. Cystatin S mRNA expression in submandibular glands of unilaterally parasympathectomized, IPR-treated rats. Quantitative analyses from five animals represented in Figure 5-2 were performed using the PhosphorImager (MolecularDynamics). The data are expressed as Light Units (Mean LU +/- SEM) and analyzed by a *t*-test for paired samples.

Effect of parasympathectomy and unilateral glossopharyngeal denervation on the weight of rat submandibular glands

Parasympathetic denervation by sectioning of the CT/LN in the rat and mouse has been shown to produce a reduction in the size of the submandibular gland and variable atrophy of different gland structures (Katsukawa *et al.*, 1990; Peronace *et al.*, 1964). The results in **Table 3** show the effect of bilateral and unilateral parasympathetic denervations on the weight of the rat submandibular glands. Bilateral parasympathectomy resulted in significant reduction of the weight of denervated glands as compared with the glands of sham-operated controls (*t*-test for independent samples; right glands, $p = 0.012$; left glands $p = 0.034$). Unilateral parasympathectomy also resulted in a significant reduction in the weight of the denervated glands as compared with the non-denervated glands (*t*-test for paired samples; $p = 0.001$; n = 5). Unilateral glossopharyngeal denervation produced a slight but consistent reduction in the weight of denervated glands as compared with the non-denervated glands. This difference was significant when the data were analyzed by the *t*-test for paired samples ($p = 0.034$; n = 5).

Morphological examination of H & E stained sections of control (non-denervated) versus parasympathectomized submandibular glands suggests a reduction in the number of acinar cells in the parasympathectomized gland (**Figure 5-6 B**) as compared with the non-denervated gland (**Figure 5-6 A**). The acini of the denervated gland, however, shows a more robust hypertrophic response to IPR

treatment than the control gland. Since cystatin S mRNA levels in the two glands are not significantly different, these results also suggest that parasympathectomy reduces the number of acinar cells, but the acinar cells become more hypertrophic and express higher levels of cystatin S gene upon IPR treatment. More detailed and quantitative analyses are required to confirm these observations and the possibility of a cause-effect relationship between hypertrophy and cystatin S gene expression.

Table 3. Effect of parasympathectomy (Px) on the weight of rat submandibular glands

Rats were IPR-treated 14 days after bilateral or unilateral denervation as described in General Methods. The data are reported in mg tissue per 100 g body weight (Mean +/- SEM). The results were analyzed by the Student's *t*-test for independent samples (bilateral denervations) or for paired samples (unilateral denervations) and the p values are indicated.

Bilateral Parasympatectomy

Glands	Sham-operated	Bilateral Px (CT/LN)	p Value
Right Gland	88.34 +/- 3.0 (n=5)	69.84 +/- 4.7(n=6)	0.012
Left Gland	86.26 +/- 2.9 (n=5)	72.15 +/- 4.6 (n=6)	0.037

Unilateral Parasympatectomy

Denervation	Non-denervated Gland	Denervated Gland	p Value
CT/LN (n=5)	101.57 +/- 3.3	74.57 +/- 0.9	0.001
Glossopharyngeal (n=5)	95.91 +/- 4.9	90.74 +/- 4.7	0.034

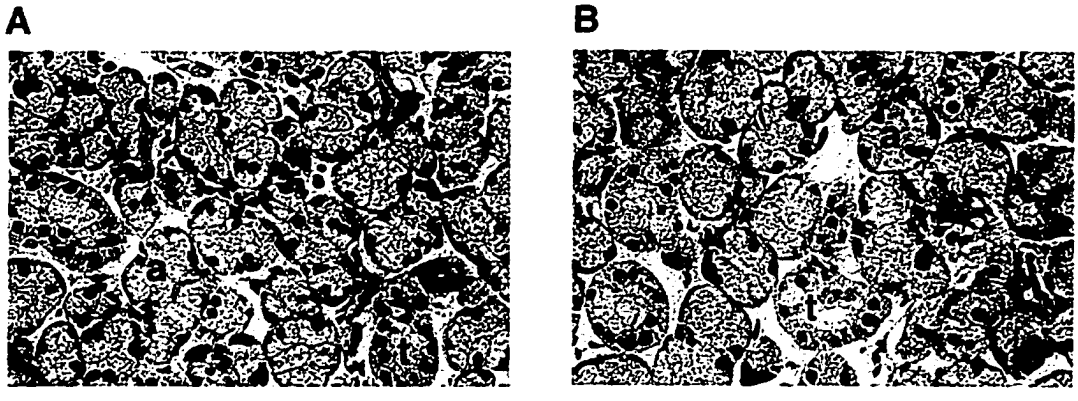


Figure 5-6. Submandibular glands of unilaterally parasympathectomized IPR-treated adult female rats, stained with Hematoxylin and Eosin (H & E). Acini (a), striated or granular convoluted tubules (t). (X125). **A.** Control (non-denervated) submandibular gland from IPR-treated rat, showing the enlargement of the acini (a) characteristic of the response to IPR. **B.** Parasympathectomized submandibular gland from the same IPR-treated rat as in **A**. Note the difference in the number of acini and the hypertrophic response in the denervated gland.

Effect of glossopharyngeal unilateral denervation on the IPR-induced expression of the rat cystatin S in submandibular glands

The results show that unilateral glossopharyngeal denervation had no effect on IPR-induced expression of cystatin S since the differences in the levels of cystatin S mRNA upon treatment with IPR in the denervated glands compared to those in the non-denervated glands were not statistically significant (*t*-test for paired samples; $p = 0.83$) (**Figures 5-7 & 5-8**).

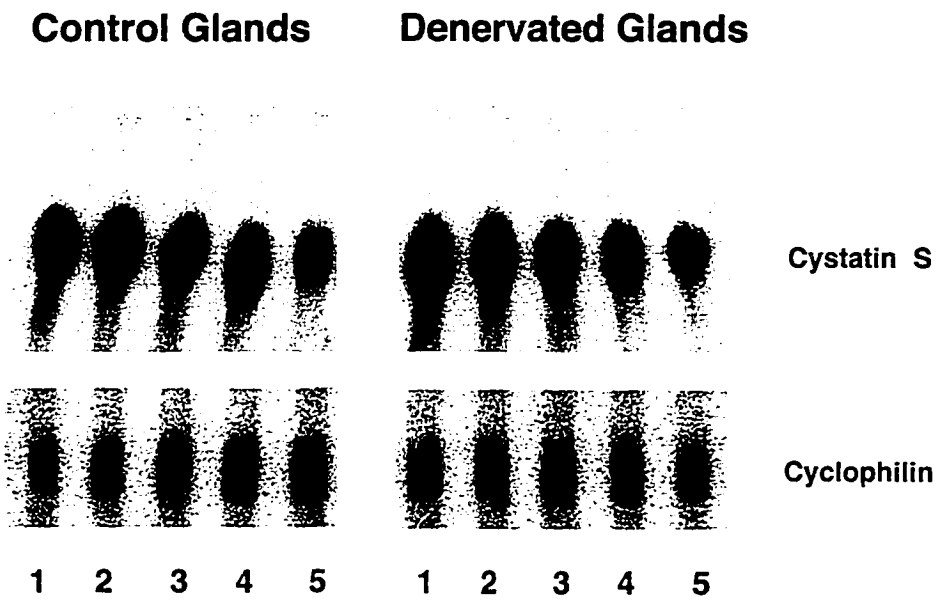


Figure 5-7. Northern blot analyses of the effect of unilateral glossopharyngeal denervation on cystatin S mRNA in submandibular glands of rats treated with IPR. Female adult rats were treated with IPR as described in Materials and Methods, 14 days after denervation. **A.** Total RNA from left glands (intact, non-denervated). **B.** Total RNA from right (denervated) glands.

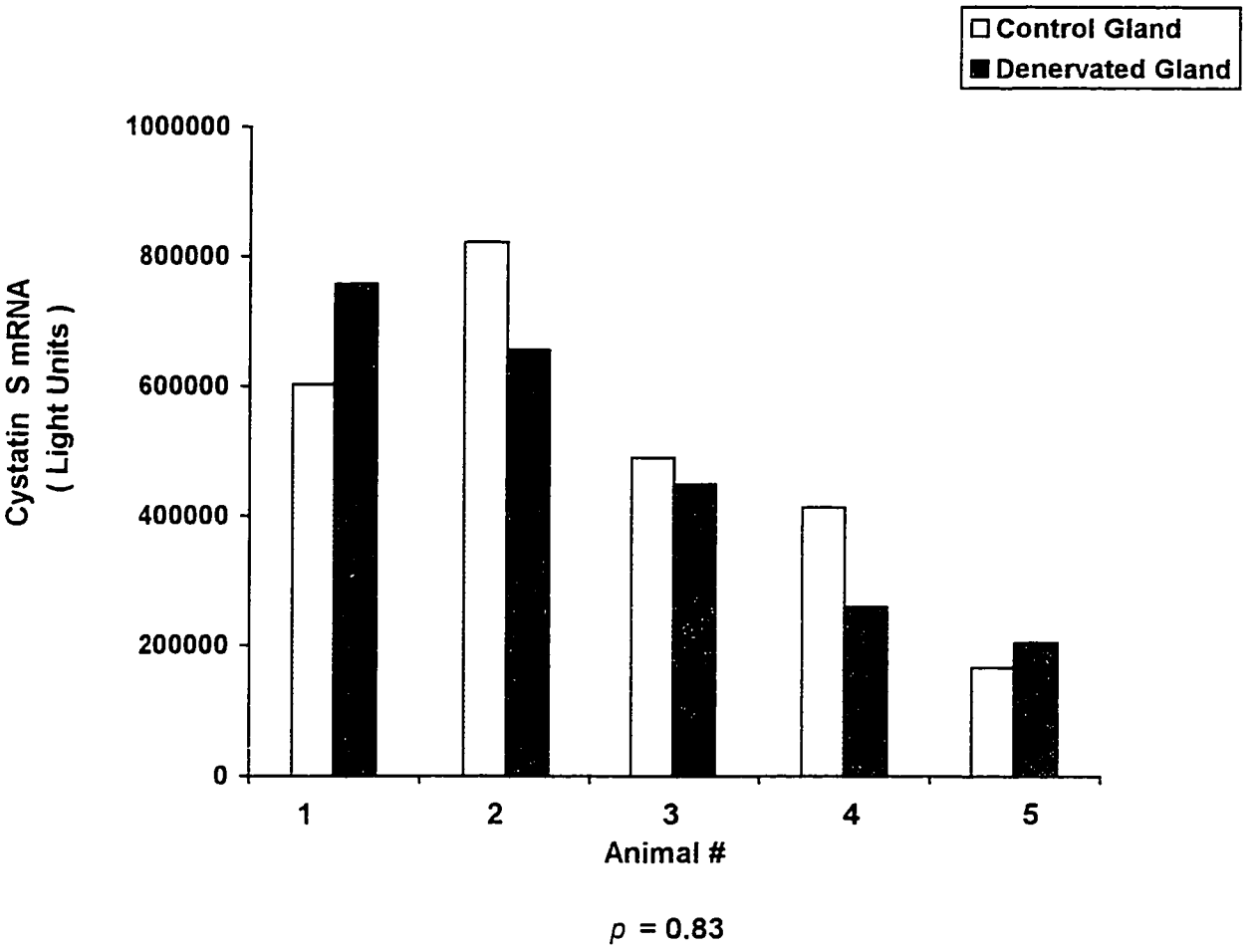


Figure 5-8. Quantitative analyses of the effect of unilateral glossopharyngeal denervation on cystatin S mRNA in submandibular glands of rats treated with IPR. Quantitative analyses was performed using the PhosphorImager (MolecularDynamics). The data from five animals represented in Figure 5-5 are expressed as Light Units (mean LU +/- SEM) and analyzed by a *t*-test for paired samples. Each pair of bars represent the control and denervated gland from the same animal.

Discussion

Previous studies have shown that the parasympathetic innervation of the rat submandibular and parotid glands regulates not only the volume and composition of saliva (Garret *et al.*, 1991; Martinez *et al.*, 1975), but also that the parasympathetic nervous system has a regulatory role in hypertrophy and hyperplasia (Katsukawa *et al.*, 1990; Peronace *et al.*, 1964; Schneyer *et al.*, 1992a; Schneyer *et al.*, 1992b; Schneyer *et al.*, 1993), and in the expression of specific genes (e.g., proline-rich proteins and β1,4-galactosyltransferase) (Humphreys-Beher and Schneyer, 1987; Schneyer *et al.*, 1992b). Interestingly, these two genes, whose expression in rat parotid glands have been shown to be downregulated by parasympathectomy (sectioning the chorda tympani nerve), seem to be regulated by β_1-adrenergic receptor-mediated mechanisms since they are induced in the parotid glands of IPR-treated rats (Ann *et al.*, 1987; Humphreys-Beher *et al.*, 1984).

The cystatin S gene seems to represent another example of the participation of parasympathetic innervation in gene regulation. Bilateral parasympathectomy reduced the expression of the cystatin S gene in the submandibular glands of IPR-treated rats, as compared to sham operated controls (**Figures 5-1, 5-2 & 5-3**). In fact, a reduction in the levels of cystatin S mRNA in the submandibular gland

could be observed when the rats were treated with IPR 3 days after bilaterally sectioning the CT/LN (**Figure 5-1 A**), and those levels were consistently, and more dramatically reduced when the animals were treated with IPR 14 days after bilateral parasympathectomy (**Figure 5-1 A, B, C and Figures 5-2 & 5-3**). These results suggest that, as in the case of the proline-rich proteins and β1,4-galactosyltransferase genes in the parotid gland, β_1-adrenergic receptor-mediated expression of the cystatin S gene in the submandibular gland requires the participation of parasympathetic regulatory factor(s).

Surprisingly, the effect of parasympathectomy on IPR-induced expression of the cystatin S gene was not observed in the glands of unilaterally denervated rats (**Figures 5-4 and 5-5**), where the levels of cystatin S mRNA were not significantly different in the denervated and non-denervated glands, regardless of the reduction of size of the denervated glands (**Table 3**). Since both the control, non-denervated gland, and the atrophic parasympathectomized gland in the same animal expressed similar levels of cystatin S mRNA in response to IPR, this suggest that the atrophy of the submandibular glands in rats bilaterally parasympathectomized (**Table 3**) is not the cause of the observed reduced expression of cystatin S gene. It has been suggested that the number of β-adrenergic receptors in rat parotid glands depends on the degree of activity of both parasympathetic and sympathetic nerves (Schneyer, *et al.*, 1986). It is not known if parasympathectomy has similar effects in the submandibular gland. It is possible

that the number of β-adrenergic receptors or their coupling to downstream elements of the signaling pathway in rat submandibular glands are more drastically affected in bilaterally than in unilaterally parasympathectomized rats and that these effects on the number and/or function of β-adrenergic receptors and not the depletion of parasympathetic factors could explain the reduction of the IPR-induced expression of the cystatin S gene in the submandibular glands of bilaterally sympathectomized rats.

There are, however, some alternative explanations. It has been suggested by estimating the size of the gland, the sensitivity to sialogogue drugs and the activity of the enzyme choline acetyltransferase, that the parasympathectomized rat submandibular gland may become reinnervated (Ohlin and Perec, 1967). Collateral innervation of parasympathectomized submandibular glands of the cat has also been documented (Emmelin and Perec, 1968). In both cases, however, the first indication of reinnervation are not seen before two months after the denervation of the glands. The recovery of fibers in the chorda tympani and lingual nerves has also been investigated in cats following nerve injury, by recording the receptor properties of the gustatory, thermosensitive and mechanosensitive units and the return of vasomotor and secretomotor responses. The combined trunk of the CT/LN was either crushed or sectioned unilaterally and the animals allowed to recover for 12 weeks. The recovery was complete after nerve crush, but significantly smaller after nerve section (Robinson, 1989). Therefore, it seems very

unlikely that significant reinnervation, if any, has occurred in the parasympathectomized submandibular glands by the time the unilaterally denervated rats were treated with IPR (14 days after the denervation), and that this could explain the lack of differences in the level of expression of the cystatin S gene. There is no evidence suggesting that reinnervation may occur after unilateral, but not after bilateral parasympathectomy of the submandibular gland and, consequently, reinnervation of the parasympathectomized glands does not seems to be a reasonable explanation for the differences in cystatin S gene expression observed in the bilaterally but not in the unilaterally parasympathectomized submandibular glands in response to IPR.

Retrograde labeling with fluorescent markers have shown variable degrees of contralateral innervation in several target organs. In young adult mice, for example, about 10 % of the sympathetic neurons of the superior cervical ganglion innervate the contralateral submandibular gland (Lahtivirta *et al.*, 1995). More recently, contralateral parasympathetic innervation has been demonstrated in the macaque monkey pupil (Kourouyan and Horton, 1997), and the lacrimal gland in the cynomolgous monkey (van der Werf *et al.*, 1996). Contralateral parasympathetic innervation of the rat submandibular gland, to the best of my knowledge, has not been documented. However, even if only very few fibers of the chorda tympani innervate the contralateral submandibular gland in the rat, they could compensate for the effect of the unilateral parasympathectomy on IPR-

induced expression of the cystatin S gene, but obviously this would not be possible in the bilaterally denervated animals. Alternatively, it is possible that parasympathetic factor(s) that participate in the regulation of cystatin S gene expression induced by IPR, may reach the denervated gland from the contralateral, non-denervated side via the circulatory system. This is a likely possibility, especially since it is known that neurocompensatory responses increasing the content of substance P and CGRP, for example, are induced in parasympathetic neurons when the contralateral side is injured (Milner *et al.*, 1997). In any case, the results show clearly that in bilaterally parasympathectomized submandibular glands the expression of the cystatin S gene induced by IPR is significatively reduced, indicating the participation of parasympathetic factor(s) in its regulation. In unilaterally denervated glands the presence of a normal parasympathetic innervation in the contralateral side could, by a still unclear mechanism, compensate for the lack of those putative factor(s) in the denervated gland, allowing normal expression of the cystatin S gene in response to IPR. This compensatory response, however, is not complete, since the denervated gland is still reduced in size compared to the non-denervated gland.

Unilateral glossopharyngeal denervation has no effect on the level of cystatin S gene expression induced by IPR. However, glossopharyngeal denervation has been reported to inhibit the expression of rat cystatin S induced by a diet containing the cysteine protease, papain (Ninomiya *et al.*, 1994). The papain

induced expression of cystatin S is also inhibited by metroprolol, a selective β_1-adrenergic receptor antagonist, suggesting that chemosensory information from the GL nerve leads, by an unknown pathway, to the activation of the β_1-adrenergic receptors in the submandibular gland (Naito *et al.*, 1992). Glossopharyngeal denervation had no effect on the IPR-induced expression of the cystatin S gene, probably because even if the circuit connecting the chemosensory information from the GL nerve with the β-adrenergic receptors is interrupted, IPR can still act directly on the β-adrenergic receptors.

CHAPTER VI

SUMMARY AND FUTURE PERSPECTIVES

1. The autonomic nervous system was shown to participate in the regulation of the cystatin S gene expression induced by the β-adrenergic agonist isoproterenol in the submandibular glands of adult female rats. This regulatory role of the autonomic nervous system was indicated by the results presented here and summarized in **Table 4,** demonstrating that:

a) Surgical bilateral or unilateral sympathetic denervation of the rat submandibular gland (by removing the superior cervical ganglion), resulted in reduction of cystatin S gene expression in response to IPR as compared to sham-operated animals.

b) Chemical sympathetic denervation, by treatment of adult female rats with reserpine, reduced the IPR-induced expression of the cystatin S gene. This effect was comparable to the reduction in expression of the cystatin S gene observed after surgical sympathectomy.

c) Surgical bilateral parasympathectomy of the submandibular gland (by severing the chorda tympani/lingual nerve) reduced the IPR-induced expression of the cystatin S gene. In contrast to the effect of unilateral sympathectomy, an effect of unilateral parasympathectomy on IPR-induced expression of the cystatin S gene was not observed in submandibular glands, may be due to a yet unexplained neurocompensatory mechanism.

d) IPR-induced cystatin S gene expression in neonatal rats was low in submandibular glands that have not yet been innervated by the sympathetic nervous system, but increased to significantly higher levels once the sympathetic nerve fibers innervated the submandibular gland. This increase in cystatin S gene expression in response to IPR did not seem not to be related to any other of the relevant changes, reported in the literature, that occur in the early developing gland, such as the differentiation of acinar cells, the increase in density of β-adrenergic receptors in the gland, or the functional coupling of the β-receptor to downstream elements of the signaling pathway (e.g., adenylate cyclase).

e) IPR did not induce expression of the cystatin S gene in primary cultures of submandibular gland cells, where the cells are obviously not in contact with sympathetic or parasympathetic nerve terminals, even if it has been reported previously that under these experimental conditions the β-adrenergic receptors on

the acinar cells remain functional and respond to IPR by increasing intracellular cAMP levels, eliciting secretory responses in rat submandibular gland cells *in vitro*.

Table 4

Effect of autonomic denervation on IPR-induced expression of the cystatin S gene in female rat submandibular glands.

Animal	Denervation	Cystatin S gene expression (% of the expression in control non-denervated glands)	Significance (p value)
Adult	Bilateral Sx [a]	47 %	0.029
	Unilateral Sx	44 %	0.005
	Chemical Sx (Reserpine)	30 %	0.024
	Bilateral Px [b]	32 %	0.027
	Unilateral Px	83 %	0.581
	Glossopharyngeal	93 %	0.830
4 day-old	Unilateral Sx [c]	76 %	0.048
8 day-old	Unilateral Sx [c]	62 %	0.023

a = **Sx,** Sympathectomy by removing the superior cervical ganglion.

b = **Px,** Parasympathectomy by severing the chorda tympani/lingual nerves.

c = Animals unilaterally sympathectomized at one day of age.

2. The present results show that cystatin S gene expression is not regulated directly by cAMP, as in the case of the early response genes (Lalli and Sassone-Corsi, 1994) since:

a) IPR-induced expression of the cystatin S gene in adult female rats was completely blocked by simultaneous treatment of the rats with cycloheximide, indicating that its expression is dependent on newly synthesized proteins.

b) It is very well documented that chemical or surgical sympathectomy produces upregulation of the β-adrenergic receptors and supersensitive responses of the submandibular gland to β-adrenergic agonists, resulting in increased levels of intracellular cAMP upon treatment with IPR. The level of cystatin S gene expression in response to IPR in sympathectomized glands did not parallel those increases in intracellular cAMP, indicating that its expression is not regulated directly via the β-receptor-cAMP signaling pathway.

3. IPR-induced expression of the cystatin S gene is not completely dependent on sympathetic or parasympathetic innervation of adult rat submandibular glands. In adult animals, surgical sympathectomy (bilateral or unilateral), chemical sympathectomy or surgical bilateral parasympathectomy reduced the expression of cystatin S gene to about the same extent, but did not block it completely.

4. IPR-induced expression of the cystatin S gene during early postnatal development of the submandibular gland is not dependent on sympathetic innervation since cystatin S mRNA was detected in the submandibular glands of IPR-treated neonatal rats before 5 days of age, when the sympathetic nerve terminals have not yet reached the gland. Similarly, cystatin S mRNA was detected after IPR-treatment in sympathectomized glands of 8-day old animals (previously denervated by removing the superior cervical ganglion of one day old rats), even if those glands have never received the sympathetic innervation. As in the case of adult animals, the absence of sympathetic innervation of the submandibular gland reduced, but did not eliminate completely, expression of the cystatin S gene in response to IPR.

The present results indicate that induction of cystatin S gene expression in the rat submandibular gland requires, in addition to the stimulation of β_1-adrenergic receptors, the participation of factor(s) from the sympathetic and the parasympathetic nervous system. Probably, the most challenging task in the design of future experiments is the identification of those factors and to unravel how they contribute to the highly regulated expression of the cystatin S gene during normal development and in response to IPR treatment. As discussed previously, there is a growing list of sympathetic and parasympathetic neuropeptides that participate in

the regulation of salivary gland function. Some of the neuropeptides identified so far are present in the sympathetic as well as in the parasympathetic nerve terminals that innervate the submandibular glands (NPY, and VIP for example) (Ekström *et al.*, 1996; Schultz, 1994; Zigmond and Sun, 1996). Some others, like SP, seem to be more exclusively parasympathetic (Virta *et al.*, 1992). Whether the sympathetic and parasympathetic factor(s) that participate in cystatin S gene expression are the same or different, is a question that will have to be addressed in future experiments. The use of commercially available neuropeptides and their antagonists will have to be considered in the design of those experiments. In this regard, the development of an *in vitro* system for the analysis of the role of different neuropeptides in the expression of the cystatin S gene may still be considered as a viable and promising experimental alternative.

It can not be inferred from the present results if the regulation of the cystatin S gene by the autonomic nervous system is exerted at the transcriptional level, as in the case of the ODC gene, or at the postranscriptional level, by reducing the half life of the message, for example. The rat submandibular gland offers a suitable experimental model for the discrimination between these alternatives.

There is another intriguing question raised by the present results: is IPR induction of the cystatin S gene mediated only by stimulation of postsynaptic β_1-adrenergic receptors on the surface of the acinar cells of the submandibular glands

or is it possible that it is acting on presynaptic β-adrenergic receptors, thus inducing the release of neurotransmitters/neuropeptides, some of which interact with each other in the complex regulation of the cystatin S gene? It is known that the effect of IPR on the expression of the cystatin S gene is prevented by β_1- but not β_2-adrenergic antagonists (Bedi, 1991a). In the results of the experiments reported by Bedi, 1991, there was a reduction of the levels of cystatin S protein in the submandibular glands of rats treated simultaneously with IPR and the β_2-adrenergic antagonist ICI-118551. Whether this reduction is the result of some nonspecific binding of the antagonist to β_1-adrenergic receptors, or is due to the blockage of presynaptic adrenergic receptors, was not addressed by the author. Peripheral sympathetic nerve terminals in many tissues are endowed with β-adrenoreceptors. Stimulation of these presynaptic receptors (by IPR, for example) results in an enhancement of neurotransmitter release from stimulated nerve terminals (Langer, 1981; Nedergaard and Abrahamsen, 1990). A careful quantitation of the effect of different β-adrenergic antagonists on IPR-induced expression of the cystatin S gene is required and would be very informative in understanding the mechanism of action of IPR and how the autonomic nervous system participates in cystatin S gene regulation.

The following is a model for how IPR may interact with neuropeptides released by sympathetic and parasympathetic nerve terminals into the

submandibular gland and induce cystatin S gene expression: $Beta_1$-adrenergic agonists induced expression of the cystatin S gene requires not only the stimulation of postsynaptic β_1-adrenergic receptors in acinar cells, increasing intracellular concentrations of cAMP and inducing the expression of specific transcription factors (c-fos, and c-jun, for example), but also the participation of a combination of neuropeptides that are released constitutively and bind to specific receptors on acinar cells. Alternatively, IPR may act also by stimulating presynaptic β-adrenergic receptors enhancing the release of neuropeptides from autonomic nerve terminals (**Figure 6-1**).

The fact that both sympathetic and parasympathetic denervation reduce the expression not only of cystatin S gene but also other salivary genes like PRP (Suares *et al.*, 1997), and that the effect of combined denervation is additive in the case of the PRP, suggest that different neuropeptides from the sympathetic and parasympathetic nervous systems involved in the regulation of salivary genes are acting synergistically. However, the possibility exist that neuropeptide(s) coming from both the sympathetic and the parasympathetic nerves that participate in the regulation of cystatin S gene expression are the same and that they need to reach a threshold concentration in order to exert their effect.

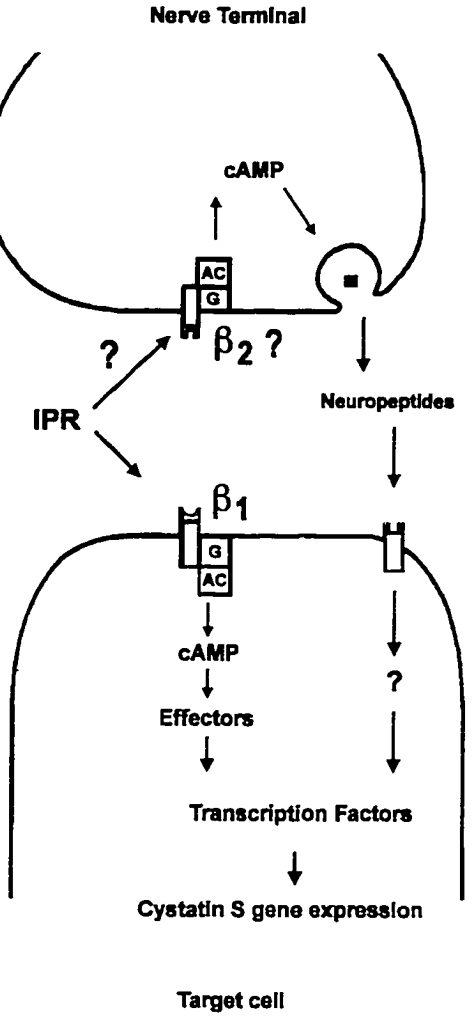


Figure 6-1. A model for the interaction of IPR with neuropeptides from the autonomic nervous system in the induction of cystatin S gene expression in the rat submandibular gland.

In summary, these studies provide evidence that the expression of the cystatin S gene in the rat submandibular gland induced by stimulation of β_1-adrenergic receptors requires not only the well documented increase in intracellular cAMP produced upon receptor stimulation by a β-adrenergic agonist but also *de novo* protein synthesis and the participation of factor(s) from the sympathetic and the parasympathetic autonomic nervous system. These results are in agreement with previous reports suggesting that some of the effects of the stimulation of β_1-adrenergic receptors on gene expression, DNA synthesis and cell proliferation in salivary glands, heart, kidney and other organs, depend on an intact autonomic innervation.

An interpretation of cystatin S physiological role and how its expression is regulated can be based on the following considerations:

1. Cystatin S gene is not expressed in the submandibular gland during early development before the sympathetic and parasympathetic nerve terminals have reached "the appropriate stage of maturation" expressing the right combination of neuropeptides. It is known that the concentration of VIP, CGRP and SP, for example, reach their maximum concentration in the submandibular gland by 4 weeks of age (Ekström *et al.*, 1994), coinciding with the time when cystatin S is maximally expressed during postnatal development (Shaw *et al.*, 1990).

2. Once the autonomic nervous system innervation of the gland has reached the appropriate stage of maturation, expression of the cystatin S gene requires the continuous stimulation of the sympathetic nervous system for a "critical period of time". This stimulation may be provided during normal development by the change from suckling and liquid diet during lactation, to masticatory activity and solid food at weaning and could allow the submandibular gland to be exposed to threshold concentrations of the required neuropeptides. From birth to about 14 days of age, rats consume liquid food (suckling period); from 15 to about 30 days they gradually adapt to solid food (weaning period) and by 20-22 days they change from a mostly milk to a mostly stock diet (Redman and Sreebny, 1976).

3. The cystatin S gene is also expressed in the submandibular gland when some "atypical" events, like continuous amputation of the lower incisor teeth (Yagil and Barka, 1986) or the presence of potentially dangerous substances (Naito *et al.*, 1992), occur in the oral cavity. This suggest that cystatin S has a protective role and that its expression is triggered when the autonomic nervous system recognizes any unusual and hazardous activity in the oral cavity. It is also possible that the increase in size of the salivary glands that usually accompanies these events is related to the requirement of massive amounts of protective proteins that need to be released into the oral cavity in order to

prevent any damage. It is not known if cystatin S has any intracellular activity and plays any functional role in acinar cell growth and proliferation.

Several human pathologies affect salivary glands. Sjögren's syndrome for example, is an autoimmune disease characterized by a progressive lymphocytic and plasma cell infiltration of salivary and lachrymal glands, leading to xerostomia and xerophtalmia (Anaya *et al.*, 1996). During and after treatments, such as head and neck radiation therapy, some degree of loss of salivary gland function is a well recognized phenomenon (Garg and Malo, 1997). The understanding of how the autonomic nervous system participates in the regulation of gene expression and salivary gland function may not only lead to the identification of key factors for salivary gland organogenesis and differentiation but may also have important practical implications in the design of new and more effective therapeutic protocols for these patients.

Finally, it has recently been suggested that salivary glands may serve as a potential target for human gene therapy and that gene transfer into salivary glands may be one rout for delivery of therapeutic substances to patients (Mastrangeli *et al.*, 1994). The salivary glands present several advantages for gene therapy: 1) The tissue is easily reached through the ducts from the oral cavity. 2) Salivary glands synthesize and secrete large quantities of proteins. 3) Synthesis and secretion of products into saliva allows continual delivery into the body through the

gastrointestinal system (reviewed in Samuelson, 1996). A better understanding of the molecular basis of salivary gene expression will be extremely useful in the development of this new approach for gene therapy.

APPENDIX

Supplementary tables. Quantitation of cystatin S gene expression

Table 5. Effect of bilateral sympathectomy on IPR-induced expression of the cystatin S gene in the submandibular glands of adult female rats (Cystatin S mRNA, Light Units).

	Sham Operated	Bilaterally Denervated
	373,197.4	151,740.1
	230,057.8	56,278.9
	233,323.4	129,174.7
	202,009.1	152,949.2
Mean	259,646.9	122,535.7
(SEM)	(38,497.0)	(22,752)

IPR was injected 14 days after denervation. Cystatin S gene expression was analyzed by Northern blot hybridization, and quantitated in the PhosphorImager (Molecular Dynamics) as described in General Methods. The data for Cystatin S gene expression are reported in Light Units.

Table 6. Effect of unilateral sympathectomy on IPR-induced expression of the cystatin S gene in the submandibular glands of adult female rats (Cystatin S mRNA, Light Units)

Animal No.	Control Gland	Sympathectomized Gland
1	48,480.7	22,339.9
2	52,474.8	17,280.8
3	49,633.4	18,706.1
4	33,992.8	34,978.6
5	37,381.1	34,100.1
6	51,990.1	27,780.4
7	48,774.2	38,555.2
8	59,731.6	21,813.7
Mean	47,807.3	26,944.4
(SEM)	(2,945.1)	(2,866.2)

IPR was injected 14 days after denervation. Cystatin S gene expression was analyzed by Northern blot hybridization and quantitated in the PhosphorImager (Molecular Dynamics) as described in General Methods. The data for cystatin S gene expression are reported in Light Units.

Table 7. Effect of chemical denervation by treatment with reserpine on IPR-induced expression of the cystatin S gene in the submandibular glands of adult female rats (Cystatin S mRNA, Light Units).

	Control	Reserpine
	270,712.8	42,172.9
	122,747.6	37,221.8
	138,420.9	39,454.0
	114,654.9	33,657.6
	107,392.7	72,782.9
Mean	150,785.8	45,057.8
(SEM)	(30,422.0)	(7,070.3)

Animals were treated with a single dose of reserpine (0.5 mg/kg body weight) for each of two days and 24 hours later with a single dose of IPR. Cystatin S gene expression was analyzed by Northern blot hybridization and quantitated in the PhosphorImager (Molecular Dynamics) as described in General Methods. The data for cystatin S gene expression are reported in Light Units.

Table 8. IPR-induced expression of the cystatin S gene during early development of the submandibular gland of female rats (Cystatin S mRNA, Light Units).

#	3 Days	4 Days	5 Days	6 Days	8 Days
1	34,781.0	35,084.0	14,205.0	41,202.0	168,009.0
2	39,432.0	31,883.0	27,967.0	34,274.0	98,726.0
3	21,326.0	56,753.0	17,766.0	34,091.0	264,152.0
4	14,931.0	22,507.0	18,224.0	23,563.0	165,721.0
5	85,009.0	40,188.0	19,302.0	16,954.0	58,074.0
6	7,619.0	41,682.0	10,913.0	19,795.0	123,225.0
7	10,260.0	112,138.0	35,964.0	24,916.0	132,539.0
8	5,287.0	42,629.0	25,896.0	45,813.0	106,369.0
9	16,215.0	22,729.0	47,837.0	16,664.0	110373.0
10	4,831.0	---------	88,722.0	11,285.0	87,119.0
Mean	23,969.1	45,072.9	30,679.6	26,865.7	131,385.7
(SEM)	(7,746.8)	(9,096.2)	(7,329.5)	(3,623.1)	(18,151.4)

Female pups of 3, 4, 5, 6 and 8 days of age were treated with a single *i.p.* injection of IPR and the expression of the cystatin S gene analyzed by Northern blots and quantitated in the PhosphorImager (Molecular Dynamics) as described in General Methods. The data for cystatin S gene expression are reported in Light Units.

Table 9. Effect of unilateral sympathectomy on the IPR-induced expression of the cystatin S gene during early development of the rat submandibular gland (Cystatin S mRNA, Light Units).

	4 Days		8 Days	
Animal No.	**Control Gland**	**Sx Gland**	**Control**	**Sx Gland**
1	509,860.4	338,713.4	1,143,042.4	735,792.4
2	376,644.4	253,433.1	713,728.9	599,833.6
3	259,373.7	206,137.1	546,438.2	181,209.1
4	489,778.0	445,729.7	1,229,616.7	752,004.0
Mean	408,914.1	311,003.3	908,206.5	567,209.8
(SEM)	(57,830.7)	(52,623.1)	(165,113.6)	(133,113.3)

Female pups of one day of age were unilaterally sympathectomized and then, treated with a single *i.p.* injection of IPR. at 4 or 8 days of age. The expression of the cystatin S gene was analyzed by Northern blots and quantitated in the PhosphorImager (Molecular Dynamics) as described in General Methods. The data for cystatin S gene expression are reported in Light Units. (**Sx**) Sympathectomized gland.

Table 10. Effect of parasympathectomy on the IPR-induced expression of the cystatin S gene in the submandibular glands of adult female rats (Cystatin S mRNA, Light Units).

	Bilateral Parasympathectomy	
	Sham operated	**Parasympathectomized**
	144,860.7	21,571.6
	80,377.0	9,654.0
	114,551.1	45,197.5
Mean	113,262.9	25,474.3
(SEM)	(18,626)	(10,444.4)

	Unilateral Parasympathectomy	
	Control Gland	**Parasympathectomized Gland**
	125,169.9	93,956.5
	92,518.8	61,334.7
	74,197.0	71,722.7
	53,310.9	52,045.5
	55,808.4	87,589.4
Mean	88,321.0	73,325.0
(SEM)	(20,480.5)	(7,834.9)

Female rats were parasympathectomized by sectioning the chorda tympani/lingual nerve and treated with a single injection of IPR 14 days after denervation. Cystatin S gene expression was analyzed by Northern blots and quantitated in the PhosphorImager (Molecular Dynamics) as described in General Methods. The data for cystatin S gene expression are reported in Light Units.

Table 11. Effect of unilateral glossopharyngeal denervation on IPR-induced expression of the cystatin S gene in the rat submandibular gland (Cystatin S mRNA, Light Units).

Animal No.	Control Gland	Denervated Gland
1	603,356.0	757,340.3
2	821,266.6	656,069.7
3	489,774.5	448,012.1
4	413,151.4	258,915.3
5	166,606.3	204,308.5
Mean	498,830.9	464,929.2
(SEM)	(107,862.5)	(107,863.3)

Female adult rats were unilaterally denervated by sectioning the glossopharyngeal nerve on the right side. The left side was an internal control. The animals were treated with a single dose of IPR 14 days after the surgery and the expression of the cystatin S analyzed by Northern blots and quantitated in the PhosphorImager (Molecular Dynamics) as described in General Methods. The data are reported as cystatin S gene expression in Light Units.

REFERENCES

Abdel-Latif, A. A., R. A. Akhtar and C. J. Zhou (1995). Effects of surgical sympathetic denervation on G-protein levels, alpha and beta-adrenergic receptors, cAMP production and adenylate cyclase activity in the smooth muscles of rabbit iris. *Curr Eye Res.* **14**(5): 405-411.

Abe, K. and C. Dawes (1978). The effects of electrical and pharmacological stimulation on the types of protein secreted by rat parotid and submandibular glands. *Archs oral Biol.* **23**: 367-372.

Anaya, J. M., H. S. McGuff, P. M. Banks and N. Talal (1996). Clinicopathological factors relating malignant lymphoma with Sjögren Syndrome. *Semin Arthritis Rheum.* **25**(5): 337-346.

Anderson, L. C., J. R. Garret, X. Zhang, G. B. Proctor and D. K. Shori (1995). Differential secretion of proteins by rat submandibular acini and granular ducts on graded autonomic nerve stimulations. *J Physiol.* **485**(2): 503-511.

Ann, D. K., S. Clements, E. M. Johnstone and D. M. Carlson (1987). Induction of tissue-specific proline-rich protein multigene families in rat and mouse parotid glands by isoproterenol. Unusual strain differences of proline-rich protein mRNAs. *J Biol Chem.* **262**(2): 899-904.

Arnett, C. D. and J. N. Davis (1979). Denervation-induced changes in alpha and beta adrenergic receptors of the rat submandibular gland. *J Pharmacol Exp Ther.* **211**(2): 394-400.

Asking, B. and J. Ekström (1979). Sensitization of the submaxillary gland of the rat after sympathetic denervation. *Acta Pharmacol Toxicol (Copenh).* **44**(5): 385-390.

Bahouth, S. W. (1992). Effects of chemical and surgical sympathectomy on expression of beta-adrenergic receptors and guanine nucleotide-binding proteins in rat submandibular glands. *Molec Pharmacol.* **42**(6): 971-981.

Barka, T. (1965a). Induced cell proliferation: the effect of isoproterenol. *Exp Cell Res.* **37**: 662-679.

Barka, T. (1965b). Stimulation of DNA synthesis by isoproterenol in the salivary gland. *Exp Cell Res.* **39**: 355-364.

Barka, T. (1980). Biologically active polypeptides in submandibular glands. *J Histochem Cytochem.* **28**: 836-859.

Barka, T., C. Yagil, H. Van Der Noen and Y. Naito (1986). Induction of the synthesis of a specific protein in rat submandibular gland by isoproterenol. *Lab Invest.* **54**(2): 165-171.

Barka, T. (1990). β–Adrenergic effects on salivary glands: growth and gene regulation. *Acta Histochem Cytochem.* **23**(2): 245-255.

Barka, T., W. W. L. Chang and H. van der Noen (1972). The effect of 6-hydroxydopamine on rat salivary glands and their response to isoproterenol. *Lab Invest.* **27**(6): 594-599.

Barka, T., R. Gubits and H. van der Noen (1986). Beta-adrenergic stimulation of c-fos gene expression in the mouse submandibular gland. *Mol Cell Biol.* **6**: 2984-2989.

Barrett, A. J. (1986). The cystatins: a diverse superfamily of cysteine peptidase inhibitors. *Biomed Biochim Acta*. **45**(11-12): 1363-1374.

Barrett, A. J. (1987). The cystatins: A new class of peptidase inhibitors. *Trends Biochem Sci*. **12**: 193-196.

Barrett, A. J. (1992). Cellular Proteolysis. An Overview. *Ann NY Acad Sci*. **674**: 1-15.

Barrett, A. J., M. E. Davies and A. Grubb (1984). The place of human gamma-trace (cystatin C) amongst the cysteine proteinase inhibitors. *Biochem Biophys Res Commun*. **120**(2): 631-636.

Barrett, A. J. and H. Kirschke (1981). Cathepsin B, cathepsin H and cathepsin L. *Methods Enzymol*. **80**: 535-561.

Bedi, G. S. (1991a). The effect of adrenergic agonists and antagonists on cysteine-proteinase inhibitor (cystatin) in rat saliva. *Arch Oral Biol*. **36**(8): 611-618.

Bedi, G. S. (1991b). The effects of autonomic drugs on the concentration of kallikrein-like proteases and cysteine-proteinase inhibitor (cystatin) in rat whole saliva. *J Dent Res*. **70**(5): 924-930.

Bedi, G. S. (1993). The effect of adrenergic agonists and antagonists on the expression of proteins in rat submandibular and parotid glands. *Crit Rev Oral Biol Med*. **4**(3-4): 565-571.

Björck, L., P. Akesson, M. Bohus, J. Trojnar, M. Abrahamson, I. Olafsson and A. Grubb (1989). Bacterial growth blocked by a synthetic peptide based on the structure of a human proteinase inhibitor. *Nature*. **337**(6205): 385-386.

Bobek, L. A. and M. J. Levine (1992). Cystatins-inhibitors of cysteine proteinases [published erratum appears in Crit Rev Oral Biol Med 1993;4(2):251]. *Crit Rev Oral Biol Med*. **3**(4): 307-332.

Borrelli, E., J. P. Montmayeur, N. S. Foulkes and P. Sassone-Corsi (1992). Signal transduction and gene control: the cAMP pathway. *Crit Rev Oncol*. **3**: 321-338.

Borth, W. (1992). a2-Macroglobulin, a multifunctional binding protein with targeting characteristics. *FASEB J*. **6**: 3345-3353.

Bottaro, B. and L. S. Cutler (1984). An electrophysiological study of the postnatal development of the autonomic innervation of the rat submandibular salivary gland. *Archs oral Biol*. **29**: 237-242.

Bradbury, N. A. and M. A. McPherson (1988). Isoproterenol-induced desensitization of mucin release in isolated rat submandibular acini. *Biochim Biophys Acta*. **970**(3): 363-370.

Brenner, G. M. and H. C. Stanton (1970). Adrenergic mechanisms responsible for submandibular salivary glandular hypertrophy in the rat. *J Pharmacol Exp Ther*. **173**(1): 166-175.

Brenner, G. M. and R. G. Wulf (1981). Adrenergic beta-receptors mediating submandibular salivary gland hypertrophy in the rat. *J Pharmacol Exp Ther*. **218**: 608-612.

Brown, W. M. and K. M. Dziegielewska (1997). Friends and Relations of the Cystatin Superfamily-New Members and Their Evolution. *Protein Science*. **6**: 5-12.

Brown-Grant, K. (1961). Enlargement of salivary glands in mice treated with

isopropylnoradrenaline. *Nature*. **191**: 1076-1078.

Brzin, J., T. Popovic, V. Turk, U. Borchart and W. Machleidt (1984). Human cystatin, a new protein inhibitor of cysteine proteinases. *Biochem-Biophys-Res-Commun*. **118**(1): 103-109.

Burnet, P. W., S. L. Eastwood and P. J. Harrison (1994). Detection and quantitation of 5-HT1A and 5-HT2A receptor mRNAs in human hippocampus using a reverse transcriptase-polymerase chain reaction (RT-PCR) technique and their correlation with binding site densities and age. *Neurosci Lett*. **178**: 85-89.

Bylund, D. B., L. R. Forte, D. W. Morgan and J. R. Martinez (1981). Effects of chronic reserpine administration on beta-adrenergic receptors, adenylate cyclase and phophodiesterase of the rat submandibular gland. *J Pharmacol Exp Ther*. **218**: 134-141.

Bylund, D. B., J. R. Martinez and D. L. Pierce (1982). Regulation of autonomic receptors in rat submandibular glands. *Molec Pharmacol*. **21**(1): 27-35.

Cardinaux, J. R. and P. J. Magistretti (1996). Vasoactive intestinal peptide, pituitary adenylate cyclase-activating peptide, and noradrenaline induced the the transcrption factors CCAAT/Enhancer Binding Protein (C/EBP)-β and C/EBPδ in mouse cortical astrocytes: involvement in cAMP-regulated glycogen metabolism. *J Neurosci*. **16**(3): 919-929.

Chang, W. W. L. (1973). Cell population changes during acinus formation in the postnatal rat submandibular gland. *Anat Rec*. **178**: 187-202.

Chang, W. W. L. and T. Barka (1973). Stimulation of acinar cell proliferation by isoproterenol in the postnatal rat submandibular gland. *Anat Rec*. **178**: 203-210.

Chaparro, O., G. W. Williams and P. A. Shaw (1994). Regulation of the cystatin S gene by steroid hormones and beta-adrenoreceptor agonists in rat submandibular glands. *Endocrine*. **2**: 841-848.

Chomczynski, P. and N. Sacchi (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*. **162**: 156-159.

Cohen, A. S. and L. A. Jones (1991). Amyloidosis. *Curr Opin Rheumatol*. **3**(1): 125-138.

Cohen, D. H., H. Feiner, O. Jensson and B. Frangioni (1983). Cerebral hemorrhage with amyloidosis (HCHWA) is related to the gastroentero-pancreatic neuroendocrine protein, gamma trace. *J Exp Med*. **158**: 623-628.

Collignon, H., C. Laborie, E. H. Tahri, A. el M'Selmi and J. M. Garel (1992). Effects of dexamethasone, calcium and 1,25-dihydroxycholecalciferol on calcitonin and calcitonin gene-related peptide mRNA levels from the CA-77 C cell line. *Thyroid*. **2**: 361-365.

Cunningham, D. D. (1992). Regulation of neuronal cells and astrocytes by protease nexin-1 and trombin. *Ann New York Acad Sci*. **674**: 228-236.

Cutler, L. S. and A. P. Chaudhry (1974). Cytodifferentiation of the acinar cells of the rat submandibular gland. *Dev Biol*. **41**(1): 31-41.

Cutler, L. S., C. P. Christian and B. Bottaro (1981). Development of stimulus-secretion coupling in salivary glands. *In: Methods in Cell Biology (Edited by Hand, A R. and Oliver, C) Academic Press, New York*. **23**: 532-545.

Cutler, L. S., C. Schneyer and C. Christian (1985). The influence of the sympathetic nervous system on the development of beta-adrenergic receptors in the rat submandibular gland. *Arch oral Biol.* **30**(4): 341-344.

Danielson, P. A., S. Forss-Petter, M. A. Brown, L. Calavetta, J. Douglass, R. J. Milner and J. G. Sutclife (1988). p1B15: A cDNA clone of the rat mRNA encoding cyclophilin. *DNA.* **7**: 261-267.

Delaisse, J. M., Y. Eckhoout and G. Vaes (1984). In vivo and in vitro evidence for the involvement of cysteine proteinase in bone resorption. *Biochem Biophys Res Commun.* **125**: 441-447.

Denny, P. C., W. D. Ball and R. S. Redman (1997). Salivary glands: a paradigm for diversity of gland development. *Crit Rev Oral Biol Med.* **8**(1): 51-75.

Devos, A., N. De Clercq, I. Vercaeren, W. Heyns, W. Rombauts and B. Peeters (1993). Structure of rat genes encoding androgen-regulated cystatin-related proteins (CRPs): a new member of the cystatin supe;family. *Gene.* **125**(2): 159-167.

Devos, A. and J. Zhangand M. Riviereand I. Vercaerenand W. Heynsand J. J. Cassimanand W. Rombautsand P. Marynenand J. Szpirerand C. Szpirer and et al. (1995). The genes coding for rat cystatin-related prostate protein (Cstrp) map to chromosome 3q41. *Cytogenet Cell Genet.* **68**(3-4): 239-242.

Domeij, S., A. Dahlqvist and S. Forsgren (1991). Enkephalin-like immunoreactivity in ganglionic cells of the larynx and superior cervical ganglion of the rat. *Regul Pep.* **32**: 95-107.

Doughney, C., R. L. Dormer and M. A. McPherson (1987). Adrenergic regulation of formation of inositol phosphates in rat submandibular acini. *Biochem J.* **2141**(3): 705-709.

Ebina, T., Y. Toya, N. Oka, J. Kawabe, C. Schwencke and Y. Ishikawa (1997). Isoform-dependent activation of adenylyl cyclase by proteolysis. *FEBS Lett.* **401**(2-3): 223-226.

Ekfors, T., W. L. Chang, R. S. Bresler and T. Barka (1972). Isoproterenol accelerates the postnatal differentiation of rat submandibular gland. *Dev Biol.* **29**: 38-47.

Ekström, J. (1979). Supersensitivity of the rat urinary bladder following "chemical sympathectomy". *Acta Pharmacol Toxicol (Copenh).* **44**(5): 377-384.

Ekström, J. (1980). Sensitization of the rat parotid gland to secretagogues following either parasympathetic denervation or sympathetic denervation or decentralization. *Acta Physiol Scand.* **108**: 253-261.

Ekström, J. (1987). Neuropeptides and secretion. *J Dent Res.* **66**: 524-530.

Ekström, J., E. Brodin, R. Ekman, R. Håkanson and F. Sundler (1984). Vasoactive intestinal polypeptide and substance P In salivary glands of the rat following denervation or duct-ligation. *Regul Pept.* **10**: 1-10.

Ekström, J., R. Ekman, R. Håkanson, A. Luts and F. Sundler (1994). Developmental studies on vasoactive intestinal peptide, substance P and calcitonin gene-related peptide in salivary glands of postnatal rats. *Acta Physiol Scand.* **151**(1): 107-115.

Ekström, J., R. Ekman, R. Håkanson, A. Luts, F. Sundler and G. Tobin (1989). Effects of capsaicin pretreatment on neuropeptides and salivary secretion of rat parotid glands. *Br J*

Pharmacol. **97**: 1031-1038.

Ekström, J., R. Ekman, R. Håkanson, S. Sjögren and F. Sundler (1988). Calcitonin gene-related peptide in rat salivary glands: neuronal localization, depletion upon nerve stimulation, and effects on salivation in relation to substance P. *Neuroscience*. **26**: 933-949.

Ekström, J., R. Ekman, A. Luts, F. Sundler and G. Tobin (1996). Neuropeptide Y in salivary glands of the rat: origin, release and secretory effects. *Regul Pept.* **61**: 125-134.

Ekström, J., B. Månsson and G. Tobin (1983). Vasoactive intestinal peptide evoked secretion of fluid and protein from rat salivary glands and the development of supersensitivity. *Acta Physiol Scand.* **119**(2): 169-175.

Elfvin, L. G., B. Lindh and T. Hokfelt (1993). The chemical neuroanatomy of the sympathetic ganglia. *Annu Rev Neurosci.* **16**: 471-507.

Elzanowski, A., W. C. Barker, L. T. Hunt and E. Seibel Ross (1988). Cystatin domains in alpha-2-HS-glycoprotein and fetuin. *FEBS-Lett.* **227**(2): 167-170.

Emmelin, N. (1964). Secretory nerves of the salivary glands. *In: Salivary Glands and Their Secretions, ed by L M Sreebny and J Meyer, Pergamon Press, New York.* : 161-173.

Emmelin, N., J. Holmberg and P. Ohlin (1965). Receptors for catecholamines in the submaxillary glands of rats. *Brit J Pharmacol.* **25**: 134-138.

Emmelin, N. and C. Perec (1968). Reinnervation of submaxillary glands after partial postganglionic denervation. *Q J l Exp Physiol.* **53**: 10-18.

Etherington, D. J. (1980). Proteinases in connective tissue breakdown. *In: Evered, G and Whelen, J (ed) Protein Degradation in Health and Disease Amsterdam: Experta Medica, Ciba Foundation Symposium.* **75**: 87-100.

Evans, C. H. (1991). The role of proteinases in cartilage destruction. *Agents Actions Suppl.* **32**: 135-152.

Fernandes-Alnemri, T. and A. Takahashiand R. Armstrongand J. Krebsand L. Fritzand K. J. Tomaselliand L. Wangand Z. Yuand C. M. Croceand G. Salvesonand W. C. Earnshawand G. Litwack and E. S. Alnemri (1995). Mch 3, a novel human apoptotic cysteine proteinase highly related to CPP32. *Cancer Res.* **55**: 6045-6052.

Finley, D. and V. Chau (1991). Ubiquitination. *Annu Rev Cell Biol.* **7**: 25-69.

Fleming, M., M. Teitelman and J. M. Sturgess (1980). The secretory response in dissociated acini from the rat submandibular gland. *J Morphol.* **163**(3): 219-230.

Garg, A. K. and M. Malo (1997). Manifestations and treatment of xerostomia and associated oral effects secundary to head and neck radiation therapy. *JADA.* **128**: 1128-1133.

Garret, J. R., A. M. Suleiman, L. C. Anderson and G. B. Proctor (1991). Secretory responses in granular ducts and acini of submandibular glands in vivo to parasympathetic and sympathetic nerve stimulation in rats. *Cell and Tissue Res.* **264**: 117-126.

Garrett, J. R. (1987). The proper role of nerves in salivary secretion: A review. *J Dent Res.* **66**: 387-397.

Gresik, E. W. (1980). Postnatal developmental changes in submandibular glands of rats and mice. *J Histochem Cytochem.* **28**(8): 860-870.

Gresik, E. W. and T. Barka (1977). The effect of neonatal sympathectomy on the response of the rat submandibular gland to isoproterenol. *J Pharmacol and Exp Ther.* **200**(1): 101-106.

Grubb, A. and H. Lofberg (1985). Human gamma-trace. Structure, function and clinical use of concentration measurements. *Scand J Clin Lab Invest Suppl.* **177**: 7-13.

Guy, B., M. Geist, K. Scott, D. Spehner, M. P. Kieny and J. P. Lecocq (1991). A specific inhibitor of cysteine proteinases impairs a Vif-dependent modification of human immunodeficiency virus type I env protein. *J Virol.* **65**: 1325-1331.

Habecker, B. A., S. E. Asmus, N. Francis and S. Landis (1996). Target Regulation of VIP Expression in Sympathetic Neurons. *Ann N Y Acad Sci.* **814**: 198-208.

Hall, H. D. and C. A. Schneyer (1964). Salivary gland atrophy in rat induced by liquid diet. *Proc Soc Exp Biol Med.* **117**: 789-793.

Hall, H. D. and C. A. Schneyer (1978). Neural regulation of compensatory enlargement of the parotid gland of the rat. *Cell Tissue Res.* **187**: 147-158.

Hanamori, T., I. J. Miller and D. V. Smith (1988). Gustatory responsiveness of fibers in the hamster glossopharyngeal nerve. *J Neurophysiol.* **60**: 478-498.

Happola, O., S. Soinila, H. Paivarinta and P. Panula (1987). [Met5]enkephalin-Arg6Phe7 and [met5]enkephalin-Arg6-Gly7-Leu8 immunoreactive fibers and neurons in the superior cervical ganglion of the rat. *Neurosci.* **21**: 283-295.

Harlow, E. and D. Lane (1988). Antibodies. A laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory.

Hausler, G., W. Haefely and H. Thoenen (1969). Chemical sympathectomy of the cat with 6-hydroxy dopamine. *J Pharmacol Exp Ther.* **170**(1): 50-61.

Hawley-Nelson, P., D. R. Roop, C. K. Cheng, T. M. Krieg and S. H. Yuspa (1988). Molecular cloning of mouse epidermal cystatin A and detection of regulated expression in differentiation and tumorigenesis. *Mol Carcinog.* **1**(3): 202-211.

Hazuda, D. J., J. Strikler, F. Kueppers, P. L. Simon and P. R. Young (1990). Processing of precursor interleukin 1-β and inflammatory disease. *J Biol Chem.* **265**(11): 6318-6322.

Hembry, R. M., M. R. Bagga, G. Murphy, B. Henderson and J. J. Reynols (1993). Rabbit models of arthritis: immunolocalization of matrix metalloproteinases and tissue inhibitors of metalloproteinase in synovium and cartilage. *Am J Pathol.* **143**(2): 628-642.

Hökfelt, T., L. G. Elfvin, M. Schulzberg, M. Fuxe, I. Said, V. Mutt and M. Goldstein (1977). Immunohistochemical evidence of vasoactive intestinal polypeptide-containing neurones and nerve fibers in sympathetic ganglia. *Neuroscience.* **2**: 885-896.

Hou, Q. C., F. E. Baker, F. J. Seidler, M. Bartolome, J. Bartolome and T. A. Slotkin (1989a). Role of sympathetic neurons in development of beta-adrenergic control of ornithine decarboxylase activity in peripheral tissues: effects of neonatal 6-hydroxydopamine treatment. *J Dev Physiol.* **11**(3): 139-146.

Hou, Q. C., J. P. Eylers, S. E. Lappi, R. J. Kavlock and T. A. Slotkin (1989b). Neonatal sympathectomy compromises development of responses of ornithine decarboxylase to hormonal stimulation in peripheral tissues. *J Dev Physiol.* **12**(4): 189-192.

Humphreys-Beher, M. and C. A. Schneyer (1986). β-Adrenergic receptors and cAMP levels of rat parotid and submandibular glands during chronic isoproterenol treatment. *J Auton Nerv Syst.* **17**: 263-271.

Humphreys-Beher, M. G. (1984). Isoprenaline-induced changes in rat parotid and submandibular glands are age-and dosage-dependent. *Biochemistry.* **221**(1): 15-20.

Humphreys-Beher, M. G., M. Immel, N. Jentoft, M. Gleason and D. M. Carlson (1984). Isolation and characterization of UDP-galactose: N-Acetylglucosamine β-galactosyltransferase activity induced in rat parotid glands treated with isoproterenol. *J Biol Chem.* **259**: 5797-5802.

Humphreys-Beher, M. G. and C. A. Schneyer (1987). Cell surface expression of 4β-galactosyltranferase accompanies rat parotid gland hypertrophy induced by changes in diet. *Biochem J.* **246**: 387-391.

Hyatt-Sachs, H., M. Bachoo, R. Schreiberg, S. A. Vaccariello and R. E. Zigmond (1996). Chemical sympathectomy and postganglionic nerve transection produce similar increases in galanin and VIP mRNA but differ in their effects on peptide content. *J Neurobiol.* **30**(4): 543-555.

Ishikawa, Y., I. Amano, T. Eguchi and H. Ishida (1995). Mechanism of isoproterenol-induced heterologous desensitization of mucin secretion from rat submandibular glands. Regulation of phosphorylation of Gi proteins controls the cell response to the subsequent stimulation. *Biochim Biopys Acta.* **1265**: 173-180.

Iwabuchi, Y. and T. Masuhara (1994a). Effects of vasoactive intestinal peptide and its homologues on the noradrenaline-mediated secretion of fluid and protein from the rat submandibular gland. *Gen Pharmacol.* **25**(7): 1427-1434.

Iwabuchi, Y. and T. Masuhara (1994b). Effects of vasoactive intestinal peptide and its homologues on the substance P-mediated secretion of fluid and protein from the rat submandibular gland. *Gen Pharmacol.* **25**(4): 705-711.

Jacoby, F. and C. R. Leeson (1959). The postnatal development of the rat submaxillary gland. *J Anat.* **93**: 201-216.

Jakubowski, M., M. Blum and J.L.Roberts (1991). Postnatal development of gonadotropin-releasing hormone and cyclophilin gene expression in the female and male rat brain. *Endocrinology.* **128**: 2702-2708.

Jarvi, R., P. Helen, M. Pelto-Huikko and A. Hervonen (1986). Neuropeptide Y (NPY)-like immunoreactivity in rat sympathetic neurones and small granule-containing cells. *Neurosci Lett.* **67**: 223-227.

Jarvinen, M., A. Rinne and V. K. Hopsu Havu (1987). Human cystatins in normal and diseased tissues-a review. *Acta Histochem.* **82**(1): 5-18.

Jensson, O., G. Gudmundsson, A. Arnason, H. Blondal, I. Petirsdottir, L. Thorteinsson, A. Grubb, H. Lofberg, D. Cohen and B. Frangione (1987). Hereditary cystatin C (gamma-trace) amyloid

angiopathy of the CNS causing cerebral hemorrhage. *Acta Neurol Scand.* **76**(2): 102-114.

Johnsson, G. and C. Sachs (1970). Effects of 6-hydroxydopamine on the uptake and storage of noradrenaline in sympathetic adrenergic neurones. *Eur J Pharmacol.* **9**: 141-155.

Kanopka, L. M., V. May and C. J. Forehand (1992). Galanin-like innervation of rat submandibular and sublingual glands: origin and effect on acinar cell membranes. *J Comp Nerobiol.* **317**: 271-282.

Katsukawa, H., Y. Ninomiya and M. Funakoshi (1990). Effects of parasympathectomy on androgen responses of the rat submandibular gland. *Archs oral Biol.* **35**(4): 273-275.

Katunuma, N. (1990). New biological functions of intracellular proteases and their endogenous inhibitors as bioreactants. *Adv Enzyme Regul.* **30**: 377-392.

Katunuma, N., H. Kakegawa, Y. Matsunaga and T. Saibara (1994). Immunological significances of invariant chain from the aspect of its structural homology with the cystatin family. *FEBS Lett.* **349**(2): 265-269.

Klimaschewski, L., I. Grohmann and C. Heym (1996). Target-dependent plasticity of galanin and vasoactive intestinal peptide in the rat superior cervical ganglion after nerve lesion and re-innervation. *Neuroscience.* **72**(1): 265-272.

Kobilka, B. (1992). Adrenergic receptors as models for G protein coupled receptors. *Annu Rev Neurosci.* **15**: 87-114.

Koide, T. and S. Odani (1987). Histidine-rich glycoprotein is evolutionarily related to the cystatin superfamily. Presence of two cystatin domains in the N-terminal region. *FEBS-Lett.* **216**(1): 17-21.

Kondo, H., M. Yamamoto, N. Yanaihara and I. Nagatsu (1988). Transient involvement of enkephalins in both the sympathetic and the parasympathetic innervations of the submandibular glands of rats. *Cell Tissue Res.* **253**: 529-537.

Konopka, L. M., V. May and C. J. Forehand (1992). Galanin-like innervation of rat submandibular and sublingual salivary glands: origin and effect on acinar cell membranes. *J Comp Neurol.* **317**(3): 271-282.

Korant, B., T. Towatari, M. Kelley, J. Brzin, B. Lenarcic and V. Turk (1988). Interactions between a viral protease and cystatins. *Biol Chem Hoppe Seyler.* **369**: 281-286.

Kourouyan, H. D. and J. C. Horton (1997). Transneuronal retinal input to the primate Edinger-Westphal nucleus. *J Comp Neurol.* **381**(1): 68-80.

Kousvelari, E., J. M. Louis, L. H. Huang and T. Curran (1988). Regulation of proto-oncogenes in rat parotid acinar cells in vitro after stimulation of β-adrenergic receptors. *Exp Cell Res.* **179**(1): 194-203.

Lah, T. T., J. Babnik, E. Schiffmann, V. Turk and U. Skaleric (1993). Cysteine proteinases and inhibitors in inflammation: their role in periodontal disease. *J Periodontol.* **64**(5 Suppl): 485-491.

Lah, T. T., J. L. Clifford, K. M. Helmer, N. A. Day, K. Moin, K. V. Honn, J. D. Crissman and B. F. Sloane (1989). Inhibitory properties of low molecular mass cysteine proteinase inhibitors

from human sarcoma. *Biochim Biophys Acta*. **993**(1): 63-73.

Lah, T. T., M. Kokalj Kunovar, L. Kastelic, J. Babnik, A. Stolfa, S. Rainer and V. Turk (1992). Cystatins and stefins in ascites fluid from ovarian carcinoma. *Cancer Lett*. **61**(3): 243-253.

Lahtivirta, S., J. Koistinaho and A. Hervonen (1995). A subpopulation of large neurones of the sympathetic superior cervical ganglion innervates the NGF-rich submandibular salivary gland in young adult and aged mice. *J Auton Nerv Syst*. **50**(3): 283-289.

Lalli, E. and P. Sassone-Corsi (1994). Signal transduction and gene regulation: the nuclear response to cAMP. *J Biol Chem*. **269**: 17359-17362.

Landis, S. C. (1990). Target regulation of neurotransmitter phenotype. *Trends Neurosci*. **13**(8): 344-350.

Langer, S. Z. (1981). Presynaptic regulation of the release of catecholamines. *Pharmacol Rev*. **32**(4): 337-362.

Levy, E., C. Lopez Otin, J. Ghiso, D. Geltner and B. Frangione (1989). Stroke in Icelandic patients with hereditary amyloid angiopathy is related to a mutation in the cystatin C gene, an inhibitor of cysteine proteases. *J Exp Med*. **169**(5): 1771-1778.

Lofberg, H. and A. O. Grubb (1979). Quantitation of gamma-trace in human biological fluids: indications for production in the central nervous system. *Scand J Clin Lab Invest*. **39**: 619-626.

Luebke, J. I. and L. L. Wright (1992). Characterization of superior cervical ganglion neurones that project to submandibular glands, the eyes, and the pineal gland in rats. *Brain Res*. **589**(1): 1-14.

Lundberg, J. M., A. Anggard, J. Fahrenkrug, T. Hokfelt and V. Mutt (1980). Vasoactive intestinal polypeptide in cholinergic neurones of exocrine glands: functional significance of coexisting transmitters for vasodilatation and secretion. *Proc Natl Acad Sci USA*. **77**: 1651-1655.

Lundberg, J. M., T. Hokfelt, A. Anggard, L. Terenius, R. Elde, K. Markey and M. Goldstein (1982a). Organization principles in the peripheral sympathetic nervous system: subdivisions by coexisting peptides (somatostatin, avian pancreatic polypeptide, and vasoactive intestinal polypeptide-like immunoreactive materials). *Proc Natl Acad Sci USA*. **79**: 1303-1307.

Lundberg, J. M., L. Terenius, T. Hokfelt, C. R. Martling, K. Tatemoto, V. Mutt, J. Polak, S. R. Bloom and M. Goldstein (1982b). Neuropeptide Y (NPY)-like immunoreactivity in peripheral noradrenergic neurons and effects of NPY on sympathetic function. *Acta Physiol Scand*. **116**: 477-480.

Maisel, A. S., J. J. Motulsky and P. A. Insel (1987). Life cycles of cardiac α and β-adrenergic receptors. *Biochem Pharmacol*. **36**: 1-6.

Mann, K. G., R. J. Jenny and S. Krishnaswamy (1988). Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Ann Rev Biochem*. **57**: 915-956.

Månsson, B., B. O. Nilsson and J. Ekström (1990). Effects of repeated infusions of substance P and vasoactive intestinal peptide on the weights of salivary glands subjected to atrophying influences in rats. *Br J Pharmacol*. **101**: 853-858.

Marks, N., M. J. Berg and M. Benuck (1986). Preferential action of rat brain cathepsin B as a peptidyl dipeptidase converting pro-opiod oligopeptides. *Arch Biochem and Biophys*. **249**: 489-499.

Martinez, J. R., D. O. Quissell, D. L. Wood and M. Giles (1975). Abnormal secretory response to parasympathomimetic and sympathomimetic stimulation from the submaxillary gland of rats treated with reserpine. *J Pharmacol Exp Ther*. **194**: 384-395.

Martins, L. M. and W. C. Earnshaw (1997). Apoptosis: alive and kicking in 1997. *Trends in Cell Biol*. 7: 111-114.

Mason, R. W. (1989). Interaction of lysosomal cystein proteases with alpha-2 macroblobulin: conclusive evidence for the endopeptidase activities of cathepsins B and H. *Arch Biochem Biophys*. **273**(2): 367-374.

Mastrangeli, A., B. O'Connell, W. Aladib, P. C. Fox, B. J. Baum and R. G. Crystal (1994). Direct in vivo adenovirus-mediated gene transfer to salivary glands. *Am J Physiol*. **266**: G1146-G1155.

Matrisian, L. M. and B. L. M. Hogan (1990). Growth factor-regulated proteases and extra-cellular matrix remodeling during mammalian development. *Curr Top Develop Biol*. **24**: 219-259.

Matsuura, S. and A. R. Hand (1991). Quantitative immunocytochemistry of rat submandibular secretory proteins during chronic isoproterenol administration and recovery. *J Histochem Cytochem*. **39**(7): 945-954.

Mehansho, H. and D. M. Carlson (1983). Induction of Protein and Glycoprotein Synthesis in Rat Submandibular Glands by Isoproterenol. *J Biol Chem*. **258**: 6616-6620.

Menaker, L., H. J. Sheetz, M. C. Cobb and J. M. Navia (1974). Gel electrophoresis of whole saliva and associated histologic changes in submandibular glands of isoproterenol-treated rats. *Lab Invest*. **30**: 341-349.

Mills, L., M. B. Hallett, M. A. MacPherson and R. L. Dormer (1993). Beta-adrenergic mobilization of Ca2+ from an intracellular store in rat submandibular acini. *Biochem J*. **293**(Pt 3): 691-695.

Milner, P., R. Crowe, A. Loesch, S. Anglin, G. Burnstock and J. R. McEcwan (1997). Neurocompensatory responses to balloon-catheter-induced injury of the rat carotid artery. *J Vasc Res*. **34**(1): 31-40.

Mirfendereski, S., G. Tobin, R. Håkanson and J. Ekström (1997). Pituitary adenylate cyclase activating peptide (PACAP) in salivary glands of the rat: origin, and secretory and vascular effects. *Acta Physiol Scand*. **160**(1): 15-22.

Montminy, M. R., G. A. Gonzalez and K. K. Yamamoto (1990a). Characteristics of the cAMP response unit. *Metabolism*. **39**(9): 6-12.

Montminy, M. R., G. A. Gonzalez and K. K. Yamamoto (1990b). Regulation of cAMP-inducible genes by CREB. *Trends Neurosci*. **13**(5): 184-188.

Mort, J. S., A. D. Recklies and A. R. Poole (1984). Extracellular presence of the lysosomal proteinase cathepsin B in rheumatoid synovium and its activity at neutral pH. *Arthritis and*

Rheum. **27**: 509-515.

Muir, T., D. Pollock and C. Turner (1975). The effects of electrical stimulation of the autonomic nerves and of drugs on the size of salivary glands and their rate of cell division. *J Pharmacol Exp Ther.* **195**: 372-381.

Murayama, N., M. Shimada, Y. Yamazoe and R. Kato (1991). Difference in the susceptibility of two phentobarbital-inducible forms, P450IIB1 and P450IIB2, to thyroid hormone- and growth hormone-induced suppression in rat liver: phentobarbital-inducible P450IIB2 suppression by thyroid hormone acting directly, but not through the pituitary system. *Mol Pharmacol.* **39**(6): 811-817.

Nagao, H., I. Matsuoka and K. Kurihara (1995). Effects of adenylyl cyclase-linked neuropeptides on the expression of ciliary neurotrophic factor-mRNA in cultured astrocytes. *FEBS Letters.* **362**: 75-79.

Naito, Y. (1981). Studies on LM protein appearing in submandibular glands of isoproterenol-treated rats. *Chem Pharm Bull (Tokyo).* **13**: 1365-1372.

Naito, Y., I. Suzuki and S. Hasegawa (1992). Induction of cystatin S in rat submandibular glands by papain. *Comp Biochem Physiol (B).* **102**(4): 861-865.

Nedergaard, O. A. and J. Abrahamsen (1990). Modulation of noradrenaline release by activation of presynaptic β-adrenoreceptors in the cardiovascular system. *Ann N Y Acad Sci.* **604**: 528-544.

Niedbala, M. J. (1993). Cytokine regulation of endotelial cell extracellular proteolysis. *Agents Act-Suppl.* **42**: 179-193.

Ninomiya, Y. and M. Funakoshi (1989). Peripheral neural basis for behavioral discrimination between glutamate and the four basic taste substances in mice. *Comp Biochem Physiol.* **92**: 371-376.

Ninomiya, Y., H. Kajiura and K. Mochizuki (1993). Differential taste responses of mouse chorda tympani and glossopharyngeal nerves to sugars and amino acids. *Neurosci Let.* **163**: 197-200.

Ninomiya, Y., H. Kajiura, Y. Naito, K. Mochizuki, H. Katsukawa and K. Torii (1994). Glossopharyngeal denervation alters responses to nutrients and toxic substances. *Physiol Behav.* **56**(6): 1179-1184.

Ohkubo, I., K. K., T. Takasawa, H. Shiokawa and M. Sasaki (1984). Isolation of a human cDNA for a thiol-proteinase inhibitor and its identity with a low molecular weight kininogen. *Biochemistry.* **23**: 5691-5697.

Ohlin, P. and C. Perec (1967). Denervation and reinnervation of the rat's submaxillary gland. *Experientia.* **23**(9): 730.

Pansky, B., D. J. Allen and G. C. Budd (1988). Review of neuroscience. New YorK, Macmillan Publishing Company.

Pennacchio, L. A., A. E. Lehesjokiand, N. E. Stone, V. L. Willourand, K. Virtanevaand, J. Miaoand, E. D'Amato, L. Ramirezand, M. Fahamand, M. Koskiniemiand, J. A. Warrington, R. Norio, A. de la Chapelle, D. R. Cox and R. M. Myers (1996). Mutations in the gene encoding cystatin B in progressive myoclonus epilepsy (EPM1). *Science.* **271**(5256): 1731-

1734.

Perec, C., F. Stefano, C. Baratti and O. Tumilasci (1975). Effects of 6-hydroxydopamine treatment at birth on the submaxillary gland of the rat. *Naunyn-Schmiedeberg's Arch Pharmacol.* **289**: 257-274.

Perec, C. J., F. J. E. Stefano and M. E. Barrio Rendo (1973). Long-lasting supersensitivity after 6-hydroxydopamine in the submaxillary gland of the rat. *J Pharmacol Exp Ther.* **186**: 220-229.

Peronace, A. A. V., T. A. Davison, A. B. Housay and C. J. Perec (1964). Alterations in submandibular and retrolingual glands following parasympathetic denervation in rats. *Anat Rec.* **150**: 25-34.

Pointon, S. E. and S. P. Banerjee (1979). Alfa-and beta-adrenergic receptors of the rat salivary gland: elevation after chemical sympathectomy. *Biochem Biophys Acta.* **584**: 231-241.

Qian, F., S. J. Chan, Q. M. Gong, A. S. Bajkowski, D. F. Steiner and A. Frankfater (1991). The expression of cathepsin B and other lysosomal proteinases in normal tissues and in tumors. *Biomed Biochem Acta.* **50**(4-6): 531540.

Quissell, D. O., K. A. Barzen and L. M. Deisher (1993). Rat submandibular and parotid protein phosphorylation and exocytosis: effect of selective cAMP analogs. *Crit Rev Oral Biol Med.* **4**(3-4): 443-448.

Quissell, D. O. and R. S. Redman (1979). Functional characteristics of dispersed rat submandibular cells. *Proc Natl Acad Sci U S A.* **76**(6): 2789-2793.

Radley, J. M. (1968). DNA synthesis in the rat submaxillary gland following injection of isoprenaline. *Aust J Exp Biol Med Sci.* **46**(6): 795-797.

Rajakumar, G. and P. J. Scarpace (1994). Relationship between cAMP production and protein secretion in rat submandibular acini: evidence for loss of reserve capacity with age. *Life Sci.* **55**(10): 761-766.

Rao, M. S. and S. C. Landis (1993). Cell interactions that determine sympathetic neurotransmitter phenotype and the neurokines that mediate them. *J Neurobiol.* **24**(2): 215-232.

Rawlings, N. D. and A. J. Barrett (1990). Evolution of proteins of the cystatin superfamily. *J Mol Evol.* **30**(1): 60-71.

Rechberger, T. and J. F. Woessner (1993). Collagenase, its inhibitors, and decoring in the lower uterine segment in pregnant women. *Am J Ostet Gynecol.* **168**(5): 15981603.

Redman, R. S. (1987). Development of salivary glands. *The Salivary System (ed Sreebny LM) CRC Press Boca Raton.* : 2-17.

Redman, R. S. and L. M. Sreebny (1976). Changes in patterns of feeding activity, parotid secretory enzymes and plasma corticosterone in developing rats. *J Nutr.* **106**: 1295-1306.

Rivett, A. J. (1993). Proteasomes. Multicatalytic proteinase complexes. *Biochem J.* **291**: 1-10.

Robinson, P. P. (1989). The reinnervation of the tongue and salivary glands after lingual nerve injuries in cats. *Brain Res.* **483**(2): 259-271.

Rosser, B. G., S. P. Powers and G. J. Gores (1993). Calpain activity increases in hepatocytes

following addition of ATP. *J Biol Chem.* **268**(3): 23593-23600.

Saido, T., H. Sorimachi and K. Suzuki (1994). Calpain: new perspectives in molecular diversity and physiological-pathological involvement. *FASEB J.* **8**(11): 814-822.

Salo, A., E. Virta and H. Uusitalo (1995). Calcitonin gene-related peptide (CGRP)and its effects on protein release in vitro in the developing submandibular gland of the rat. *Regul Pept.* **55**(2): 155-165.

Samuelson, L. C. (1996). Transgenic approaches to salivary gland research. *Annu Rev Physiol.* **58**: 209-229.

Schneyer, C. (1972). Regulation of salivary gland size. *In Regulation of Organ and Tissue Growth, ed by R Goss, Academic Press, New York, N Y.* : 211-232.

Schneyer, C. A. (1970). Mitosis induced in adult rat parotid following normal activity of the gland. *Proc Soc Exp Biol Med.* **134**: 98-102.

Schneyer, C. A., M. Humphreys-Beher and H. D. Hall (1986). Beta-adrenoreceptors in the rat parotid gland enlarged by salivariectomy and bulk diet. Effects of denervation. *J Auton Nerv Syst.* **17**(3): 165-173.

Schneyer, C. A., M. Humphreys-Beher and H. D. Hall (1988). Comparison of effects of surgical and chemical sympathectomy on b adrenergic and muscarinic receptors of parotid gland of young and adult rats. *Proc Soc Exp Biol Med.* **188**: 420-426.

Schneyer, C. A., M. Humphreys-Beher and H. D. Hall (1992a). Nerve Growth Factor Effects on Inhibition by Submandibular Gland Ablation or Autonomic Denervation of Activity-Induced Parotid Growth. *J Auton Nerv Syst.* **37**: 137-144.

Schneyer, C. A., M. G. Humphreys-Beher and H. D. Hall (1992b). Effects of selective denervation and nerve growth factor on activity-mediated growth of rat parotid gland. *Proc Soc Exper Biol and Med.* **200**: 127-132.

Schneyer, C. A., M. G. Humphreys-Beher, H. D. Hall and D. Jirakulsomchok (1993). Mitogenic activity of rat salivary glands after electrical stimulation of parasympathetic nerves. *Am J Physiol.* **264**(Gastrointest. Liver Physiol. 27): G935-G938.

Schneyer, C. A. and J. M. Shackleford (1963). Accelerated development of salivary glands of early postnatal rats following isoproterenol. *Proc Soc Exp Biol Med.* **112**: 320-324.

Schotzinger, R., X. Yin and S. Landis (1994). Target Determinations of Neurotransmitter Phenotype in Sympathetic Neurons. *J Neurobiol.* **25**(6): 620-639.

Schultz, T., Soinila, J., Tolonen, R., Happola, O., Uusitalo, H., and Salo, A. (1994). The sympathetic and parasympathetic nature of neuropeptide Y-immunoreactive nerve fibers in the major salivary glands of the rat. *Histochem J.* **26**(7): 563-570.

Scott, G. K. (1992). Proteinases and proteinase inhibitors as modulators of animal cell growth. *Comp Biochem Physiol (B).* **103**(4): 785-793.

Scott, G. K. and C. A. Tse (1992). Growth-related proteinases in cultured human tumor cells. *Biol Chem Hoppe Seyler.* **373**(7): 605-609.

Segawa, A., S. Terakawa, S. Yamashina and C. R. Hopkins (1991). Exocytosis in living salivary glands: direct visualization by video-enhanced microscopy and confocal laser microscopy. *Eur J Cell Biol.* **54**(2): 322-330.

Selye, H., R. Veilleux and M. Cantin (1961). Excessive stimulation of salivary gland growth by isoproterenol. *Science (Washington).* **133**: 44-45.

Sharkey, K. A., R. Mathison, M. N. Sharif and J. S. Davison (1989). The effects of strepzotocin-induced diabetes on the peptidergic innervation and function of the rat parotid gland. *J Auton Nerv Syst.* **27**: 127-138.

Shaw, P. A. and T. Barka (1989). Beta-adrenergic induction of a cysteine-proteinase-inhibitor mRNA in rat salivary glands. *Biochem J.* **257**(3): 685-689.

Shaw, P. A., T. Barka, A. Woodin, B. S. Schacter and J. L. Cox (1990). Expression and induction by β-adrenergic agonists of the cystatin S gene in submandibular glands of developing rats. *Biochem J.* **265**(1): 115-120.

Shaw, P. A., J. L. Cox, T. Barka and Y. Naito (1988). Cloning and sequencing of cDNA encoding a rat salivary cysteine proteinase inhibitor inducible by β-adrenergic agonists. *J Biol Chem.* **263**(34): 18133-18137.

Sheetz, J. H. and L. Menaker (1979). Morphological and functional study of the effect of isoproterenol on salivary gland cells. *Cell Tissue Res.* **203**(2): 321-329.

Shepherd, G. M. (1988). Neurobiology. New YorK, Oxford University Press.

Shikama, N., Lyon, J., and La Thangue, N. B. (1997). The p300/CBP family: integrating signals with transcription factors and chromatin. *Trends Cell Biol.* **7**: 230-236.

Sloane, B. and K. Honn (1984). Cysteine proteinases and metastasis. *Cancer Metast Rev.* **3**: 249-263.

Sloane, B. F., Rozhin, J., Robinson, D., and Honn, K.V. (1990). Role for cathepsin B in tumor growth and progression. *Biol Chem Hoppe Seyler (Suppl).* **371**: 193-198.

Slotkin, T. A., B. A. Lorber, E. C. McCook, G. A. Barnes and F. J. Seidler (1995). Neural input and the development of adrenergic intracellular signaling: neonatal denervation evokes neither receptor upregulation nor persistent supersensitivity of adenylate cyclase. *Dev Brain Res.* **88**: 17-29.

Snell, R. S. (1992). Neuroanatomy: A review with questions and explanations. Boston, Little, Brown and Company.

Sorg, O. and P. J. Magistretti (1992). Vasoactive intestinal peptide and noradrenaline exert long-term control of glycogen levels in astracytes: blockage by protein synthesis inhibition. *J Neurosci.* **12**: 4923-4931.

Sousa Neto, J. A., A. Seidel, L. Vollrath and B. Manz (1990). Synaptic ribbons of the pineal gland: responses to in-vivo and in-vitro treatment with inhibitors of protein synthesis. *Cell Tisssue Res.* **260**(1): 63-67.

Sporn, J. R., T. K. Harden, B. B. Wolfe and P. B. Molinoff (1976). β-Adrenergic receptor involvement in 6-hydroxidopamine-induced supersensitivity in rat cerebral cortex. *Science.*

194(4265): 624-626.

Stefano, F. and C. Perec (1981). Denervation supersensitivity in salivary glands. *Trends Pharmacol Sci*. **2**: 107-109.

Stoeckle, M. Y. and L. Guan (1993). Improved resolution and sensitivity of Northern blots using polyacrylamide-urea gels. *BioTechniques*. **15**: 227-231.

Stracke, M. L. and L. A. Liotta (1992). Multi-step cascade of tumor metastasis. *In vivo*. **6**(4): 309-316.

Suares, J. C., D. K. Shori, Asking, B. and G. B. Proctor (1997). Reduced expression of proline-rich proteins in denervated rat parotid glands. *Biochem Soc Trans*. **25**: 33S.

Suzuki, K., T. C. Saido and S. Hirai (1992). Modulation of cellular signals by calpain. *Ann N Y Acad Sci*. **674**: 218-227.

Teitelman, G., T. Baker, H. Joh and D. J. Reis (1979). Apparance of catecholamine synthesizing enzymes during development of the rat nervous system: Possible role of tissue environment. *Pro Natl Acad Sci USA*. **76**: 509-513.

Thomas, D. J., A. D. Richards and J. Kay (1989). Inhibition of aspartic proteinases by alpha 2-macroglobulin. *Biochem J*. **259**(3): 905-907.

Trabandt, A., R. E. Gay, H. G. Fassbender and S. Gay (1991). Cathepsin B in synovial cells at the site of joint destruction in rheumatoid arthritis. *Arth Rheum*. **34**(11): 1444-1451.

Tranzer, J. P., and Thoenen, H. (1968). An electron microscopic study of selective, acute degeneration of sympathetic nerve terminals after administration of 6-hydroxydopamine. *Experientia*. **24**: 155-156.

Turk, V., J. Brzin, M. Kotnik, B. Lenarcic, T. Popovic, A. Ritonja, M. Trstenjak, L. Begic Odobasic and W. Machleidt (1986). Human cysteine proteinases and their protein inhibitors stefins, cystatins and kininogens. *Biomed Biochim Acta*. **45**(11-12): 1375-1384.

Turk, B., V. Turk and D. Turk (1997). Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. *Biol Chem*. **378**(3-4): 141-150.

Twining, S. S. (1994). Regulation of Proteolytic Activity in Tissues. *Crit Rev Biochem Mol Biol*. **29**(5): 315-383.

Tyrrel, S. and S. C. Landis (1994). The appearance of NPY and VIP in sympathetic neuroblast and subsequent alterations in their expression. *J Neurosci*. **14**(7): 4529-4547.

van der Werf, F., B. Baljet, M. Prins and J. A. Otto (1996). Innervation of the lacrimal gland in the cynomolgous monkey: a retrograde tracing study. *J Anat*. **188**(Pt 3): 591-601.

Virta, E., A. Salo and H. Uusitalo (1994). Substance P (SP) and neurokinin A (NKA) in developing submandibular glands of the rat. *Int J Neurosci*. **12**(3): 175-183.

Virta, E., J. Tornwall and H. Uusitalo (1992). Substance P and neurokinin A immunoreactive nerve fibres in the developing salivary glands of the rat. *Histochemistry*. **98**(5): 317-325.

Wagner, J. P., F. J. Seidler, S. E. Lappi, E. C. McCook and T. A. Slotkin (1995). Role of presynaptic input in the ontogeny of adrenergic cell signaling in rat brain: β receptors,

adenylate cyclase and c-fos protoncogene expression. *J Pharmacol Exp Ther.* **273**(1): 415-426.

Weiss, B., L. H. Greengerg and H. Cantor (1980). Denervation supersensitivity and β-adrenergic receptors as a function of age. *Adv Biochem Psychopharmacol.* **21**: 461-472.

Wells, H. (1963). The neural regulation of salivary gland growth. *Ann NY Acad Sci.* **106**: 654-667.

Werb, Z., C. M. Alexander and R. R. Adler (1992). Expression and function of matrix metalloproteinases in development. *Matix Suppl.* **1**: 337-343.

Wessels-Reiker, M., R. Baisiboina, A. C. Howlett and R. Strong (1993). Vasoactive intestinal polypeptide-related peptides modulate tyrosine hydroxilase gene expression in PC12 cells through multiple adenylate cyclase-coupled receptors. *J Neurochem.* **60**: 1018-1029.

Woessener, J. F. (1991). Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J.* **5**(8): 2145-2154.

Yagil, C. and T. Barka (1986). Induction of a specific (LM) protein in the submandibular gland of the rat by repeated amputation of the lower incisor teeth. *Am J Anat.* **177**(4): 513-518.

Yamada, S., H. I. Yamamura and W. R. Roeske (1980). Alterations in cardiac autonomic receptors following 6-hydroxydopamine. *Mol Pharmacol.* **18**: 185-192.

Yamada, S., H. I. Yamamura and W. R. Roeske (1986). Effects of chemical sympathectomy with 6-hydroxydopamine on α-and β-adrenoreceptors and muscarinic cholinoreceptors in rat kidney. *Eur J Pharmacol.* **121**: 345-353.

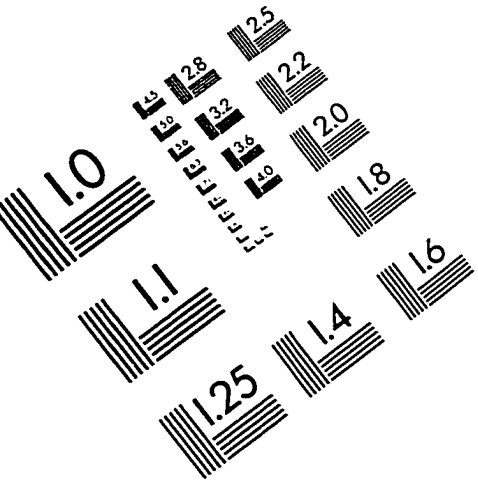
Yamashina, S. and T. Barka (1972). Localization of peroxidase activity in the developing submandibular gland of normal and isoproterenol-treated rats. *J Histochem Cytochem.* **20**(11): 855-872.

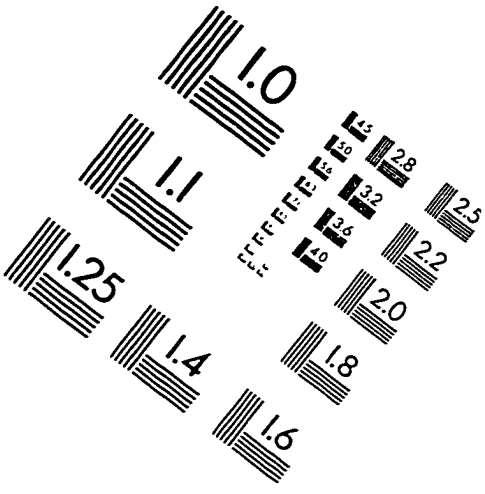
Zia, H., T. Hida, S. Jacowlew, M. Birrer, Y. Goyes, J. C. Reubi, M. Fridkin, I. Gozes and T. W. Moody (1996). Breast cancer growth is inhibited by vasoactive intestinal peptide (VIP) hybrid, a synthetic VIP receptor antagonist. *Cancer Res.* **56**(15): 3486-3489.

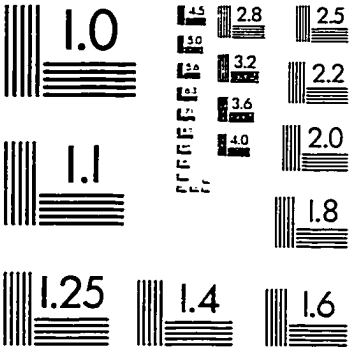
Ziff, E. B. (1990). Transcription factors: a new family gathers at the cAMP response site. *Trends Genet.* **6**: 69-72.

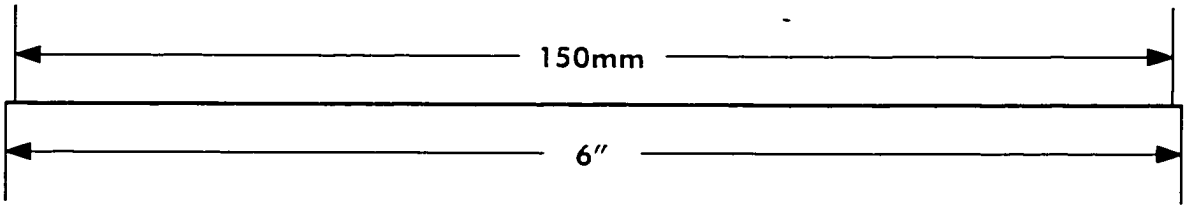
Zigmond, R. E. and Y. Sun (1996). Regulation of neuropeptide expression in sympathetic neurons. Paracrine and retrograde influences. *Ann N Y Acad Sci.* **814**: 181-197.

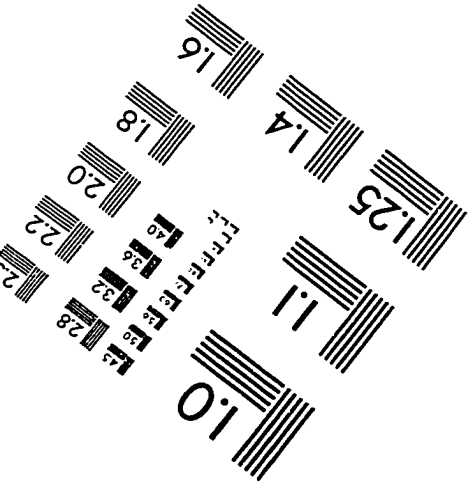
IMAGE EVALUATION TEST TARGET (QA-3)











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