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Expression and induction by β-adrenergic agonists of the cystatin S gene in submandibular glands of developing rats

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Repeated administration of the β-adrenergic agonist isoprenaline (isoproterenol, IPR), which produces hypertrophic/hyperplastic enlargements of rat submandibular and parotid glands, induces synthesis of a secretory protein shown to be a cysteine proteinase inhibitor, rat cystatin S. In the current study, Northern blot and hybridizations *in situ* were carried out to establish the developmental and β-adrenergic regulation of the expression of the cystatin S gene. Cystatin S mRNA was not detected in submandibular glands of 20-day-old fetuses, nor in the glands of newborn or 10-day-old rats. However, steady-state levels of cystatin S mRNA increased between 21 and 28 days, reaching a conspicuously high concentration at 28 days; cystatin S mRNA then declined rapidly to a barely detectable level in glands of 32-day-old rats. IPR administration for 4 days induced high levels of cystatin S mRNA in submandibular glands of developing and adult rats. In both prepubertal and mature animals, induction of cystatin S mRNA in submandibular glands was more pronounced in female than in male animals. Hybridizations *in situ* revealed cystatin S mRNA only in acinar but not in duct cells of the submandibular gland. Developmentally, expression of the cystatin S gene coincided with acinar cell differentiation. These data suggest a complex neural, hormonal and developmental regulation of salivary cystatin genes.

INTRODUCTION

β-Adrenergic agonists, known to influence the differentiation, growth and function of salivary glands, induce the expression of a number of genes in these organs. These include the genes encoding the family of proline-rich proteins in the salivary glands of mice, rats, hamsters and the subhuman primate *Macaca fascicularis* (Mehansho *et al.*, 1984; Ziemer *et al.*, 1984; Belford *et al.*, 1984; Ann *et al.*, 1987), the proto-oncogene c-*fos* (Barka *et al.*, 1986), and the gene encoding the enzyme 4β-galactosyltransferase (Humphreys-Beher, 1988). A secretory protein that, in rat submandibular glands, is highly inducible by the β-adrenergic agonist isoprenaline (isoproterenol, IPR) has been described by Menaker *et al.* (1974) and partially characterized by Naito and his coworkers (Naito, 1981; Naito & Saito, 1982). Recently we cloned and sequenced cDNAs encoding this protein, previously called LM protein, and have established that it is a cysteine proteinase inhibitor belonging to family 2 of the cystatin superfamily of mammalian cysteine proteinase inhibitors (Shaw *et al.*, 1988). This inhibitor has been named rat cystatin S. The expression of the cystatin S gene in submandibular glands of adult rats is induced by IPR (Shaw & Barka, 1989). The most pronounced accumulation of cystatin S mRNA was observed in submandibular glands of rats treated with the agonist for several days; its increase in concentration paralleled the increase in the level of the protein cystatin S.

IPR also greatly stimulates DNA synthesis and mitotic activity in the submandibular and parotid glands of rats and mice (Barka, 1965; Baserga & Heffler, 1967). This stimulation is sex-dependent; it is much greater in female than in male rats (Barka, 1967). IPR stimulation of DNA synthesis is also age-dependent, and it is inversely correlated with the proliferative activity in unstimulated submandibular glands of developing animals. Maximal stimulation of DNA synthesis by a single dose of the drug was observed in young adult animals in which the proliferative activity in the glands declined to a low level characteristic of the adult gland (Barka *et al.*, 1973). The observation of this sex- and age-dependent response of salivary glands to the growth-stimulatory effects of IPR prompted an analysis of IPR induction of cystatin S gene expression in the two sexes and during postnatal development of the rat.

MATERIALS AND METHODS

Treatment of rats

Sprague–Dawley rats 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. DL-IPR hydrochloride was dissolved in 0.1% (w/v) sodium metabisulphite in 0.85% (w/v) NaCl. In all experiments, 0.1 μmol of IPR/g body wt. was injected intraperitoneally once daily for 4 days. The animals were killed under diethyl ether anaesthesia 24 h after the last injection of the drug. For the sexual dimorphism studies, adult (150–180 g) and 10-day-old rats were used. For the developmental studies, groups of female rats of 5, 15, 16, 20, 23, 27 and 30 days of age were injected with IPR and killed, together with age-matched controls, 24 h after the last injection of the drug. Therefore, the final ages of the rats examined were 10, 20, 21, 25, 28, 32 and 35 days. The experiments were performed

Abbreviation used: IPR, isoprenaline (isoproterenol; 4-{1-hydroxy-2-[(1-methylethyl)amino]ethyl}benzene-1,2-diol).

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three times. The control and experimental animals were littermates, and there were at least five rats pooled in each group. For studies on isolated RNA, the submandibular glands (without the sublingual glands) were removed and immediately frozen in liquid N_2. For hybridization studies *in situ*, submandibular and sublingual glands were embedded in Histo Prep tissue-embedding medium and quenched in isopentane cooled with liquid N_2. As a negative control for the hybridization studies *in situ*, pieces of liver were treated similarly.

Northern and slot-blot hybridization

Hybridization probes were prepared by transcription of a cRNA probe from the recombinant plasmid pGEM-3 containing cDNA encoding the rat cystatin S protein sequence, part of the signal peptide sequence, and all of the 3′-untranslated sequence (Shaw *et al.*, 1988). All RNA samples were analysed by at least three separate Northern blot hybridizations. Total RNA (20 μg) and poly(A)$^+$ RNA (1 μg from the submandibular glands of rats treated with IPR for 6 days, i.e. chronic treatment), a positive control, were electrophoresed through 1.5% (w/v) agarose/6% (v/v) formaldehyde gels (Rave *et al.*, 1979). Nitrocellulose blots of the RNA were prepared (Thomas, 1980) and hybridized at 65 °C for 16 h to ^{32}P-labelled rat cystatin S cRNA (10^6 c.p.m./lane; 2.7×10^8 c.p.m./μg) using the conditions of Wahl *et al.* (1979). The blots were washed as previously described (Shaw *et al.*, 1988).

Slot-blot analyses were performed to determine the relative abundance of IPR-induced cystatin S mRNA in male and female submandibular glands. A dilution series of RNA was made beginning with 2.5 μg of RNA in 150 μl of water. Formamide was added to the RNA samples to a final concentration of 50% (v/v), and SSC (1 × SSC = 0.15 M-NaCl/0.015 M-sodium citrate) to a final concentration of 2 ×. The samples were heated for 10 min at 65 °C, cooled on ice, applied to nitrocellulose sheets using a Schleicher and Schuell slot-blotting apparatus (Keene, NH, U.S.A.), and hybridized as described above. After a 12–18 h exposure of the blot to X-ray film, films were developed and the fold differences in hybridization intensities were estimated by scanning with a densitometer (Bio-Rad Videodensitometer, model 620). Three different regions of each slot were scanned and the areas were computed. The mean areas and S.D. values were calculated for each dilution of RNA. The relative abundance of IPR-induced cystatin S mRNA was calculated using the linear standard curve of the male.

Hybridization *in situ*

The protocol of Shivers *et al.* (1986) was modified as described below for the detection of cystatin S mRNA in histological sections. (1) The sections (10 μm thick) were mounted on to gelatin-coated glass slides that were additionally coated by a brief soaking in 0.02% (w/v) poly-L-lysine, then rinsed with water and dried at 60 °C overnight. This treatment enabled us to carry out the hybridizations at 52 °C for 18 h with no loss of tissue from the slides. (2) Before prehybridization, tissue sections were treated with proteinase K [1 μg/ml in 10 mM-Tris/HCl (pH 8)/50 mM-EDTA] for 5 min, then rinsed in 2 × SSC and air dried. (3) After hybridization with an ^{35}S-labelled cRNA probe (approx. 1000 c.p.m./μl of buffer), slides were rinsed in 500 mM-NaCl/20 mM-sodium phosphate (pH 6.7)/0.05% (w/v) sodium pyrophosphate/14.7 mM-β-mercaptoethanol/1% (w/v) sodium thiosulphate at room temperature, treated for 30 min at 37 °C with 20 μg of RNAase A/ml in 500 mM-NaCl/10 mM-Tris/HCl (pH 8.0)/1 mM-EDTA, and rinsed in the same buffer without RNAase. The sections were washed for 3 h at 52 °C in 50 mM-NaCl/20 mM-sodium phosphate (pH 6.7)/14.7 mM-β-mercaptoethanol/0.05% (w/v) sodium pyrophosphate/1% (w/v) sodium thiosulphate, and for an additional 24 h in the same buffer at room temperature. They were dehydrated through 70% ethanol containing 300 mM-ammonium acetate, 95% ethanol containing 300 mM-ammonium acetate and 100% ethanol. The dry sections were exposed to X-ray film at −80 °C for 2.5 days, and were then dipped into Kodak NTB2 emulsion diluted 1:1 with 600 mM-ammonium acetate (pH 7.1). Exposure time was 4–8 days. After development, the sections were lightly stained with Cresyl Violet.

RESULTS

Cystatin S mRNA was not detectable by Northern blot hybridization of total RNA extracted from submandibular glands of untreated adult male or female rats (Fig. 1, lanes 1 and 3). IPR-induced steady-state levels of

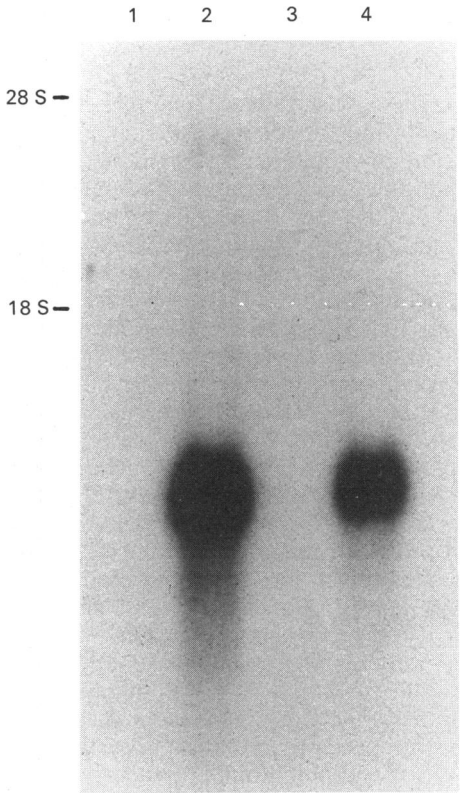

Fig. 1. Northern blot analysis of cystatin S mRNA in the submandibular glands of adult rats

IPR was given for 4 days and the RNA was extracted 24 h after the last injection of the drug. Northern blot hybridization of total submandibular gland RNA (20 μg/lane) from adult rats was carried out as described in the Materials and methods section. Lane 1, control female; lane 2, IPR-treated female; lane 3, control male; lane 4, IPR-treated male.

Table 1. Effect of IPR on cystatin S mRNA levels in adult and developing rat submandibular glands

Slot-blot hybridizations were scanned as outlined in the Materials and methods section and the relative abundance of each sample was determined using the linear standard curve of the male (means ± S.D.).

Animal	Relative abundance of IPR-induced cystatin S mRNA
Adult male	1.00
Adult female	2.85 ± 0.49
Female (10 days)	3.83 ± 0.29
Female (20 days)	4.26 ± 0.10
Female (25 days)	2.51 ± 0.89
Female (32 days)	0.74 ± 0.22

the 900 bp cystatin S mRNA were higher in the glands of adult females than of adult males (Fig. 1, lanes 2 and 4). Indeed, the relative abundance of cystatin S mRNA, as analysed by slot-blot hybridization, indicated that the concentration of cystatin S mRNA was approx. 3-fold higher in the submandibular glands of IPR-treated female than in IPR-treated male rats (Table 1). The sex difference in IPR-induced cystatin S mRNA was also observed in prepubertal animals. The abundance of steady-state cystatin S mRNA was greater in the glands of IPR-treated 15-day-old female rats than in identically treated 15-day-old male rats (Fig. 2, lanes 2 and 4).

Next we analysed the expression of the cystatin S gene in submandibular glands of normally developing animals. Steady-state cystatin S mRNA was not detected by Northern blot analyses of total RNA in the submandibular glands of fetal, newborn or 10-day-old rats (Fig. 3, lanes 1, 2 and 3). The concentration of cystatin S mRNA increased between 21 to 28 days, reaching a maximum at 28 days (Fig. 3, lanes 4 and 5), and declining at 32 days (Fig. 3, lane 6).

IPR-induction of rat cystatin S mRNA during postnatal development was examined by injection of groups of rats as described in the Materials and methods section. As expected, submandibular gland weights were dramatically increased by the administration of IPR. The greatest difference was observed in 20-day-old rats, in which the average gland weight was doubled (Table 2). At each age of postnatal development studied, IPR administration led to a dramatic increase in the concentration of cystatin S mRNA (Fig. 4, lanes 3, 4, 5 and 6). Slot-blot hybridization studies indicated an age-dependency of IPR-induced cystatin S mRNA. Submandibular glands from 10- and 20-day-old IPR-treated animals had higher cystatin S mRNA levels than glands from identically treated 25- and 32-day-old animals (Table 1). In addition, the relative abundance of IPR-induced rat cystatin S mRNA was slightly greater in the 10- and 20-day-old rats than in the adult females (Table 1).

Previous immunocytochemical studies revealed that, with the exception of some scattered cells in the duct system, IPR-induced cystatin S (LM protein) was localized in acinar cells in adult rat submandibular glands, suggesting that the protein is synthesized by these cells (Barka *et al.*, 1986). In postnatally developing rats, the

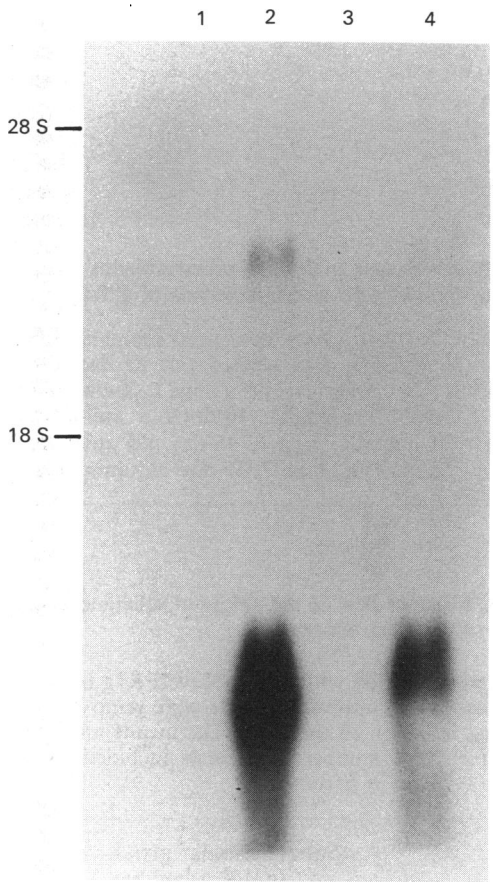

Fig. 2. Northern blot analysis of cystatin S mRNA in the submandibular glands of 15-day-old rats

Northern blot hybridization was performed with a ^{32}P-labelled cRNA probe representing the 3′-untranslated end of rat cystatin S mRNA. Total submandibular gland RNA (20 μg/lane) from 15-day-old rats was electrophoresed and blotted on to nitrocellulose as described in the Materials and methods section. Lane 1, control female; lane 2, IPR-treated female; lane 3, control male; lane 4, IPR-treated male.

protein was demonstrable in the pro-acinar and acinar cells, but not in the terminal tubule or duct cells (Yagil *et al.*, 1986). Hybridizations *in situ*, using an ^{35}S-labelled cRNA probe specific for rat cystatin S mRNA, demonstrated that the site of synthesis of cystatin S mRNA is primarily the acinar cells in the glands of IPR-treated adult rats (Fig. 5). High concentrations of cystatin S mRNA were revealed in acinar cells, whereas various segments of the duct system showed no specific signals. In accord with Northern blot hybridization data, the concentration of mRNA was higher in acinar cells in submandibular glands of adult females than in the glands of adult male animals (Figs. 5*b* and 5*d*). No specific signals were observed in any structures in the sublingual gland, which is closely attached to the submandibular gland, nor in the liver, used as a negative control (results not shown). In the submandibular glands of IPR-treated developing female rats, hybridization *in situ* revealed high concentrations of cystatin S mRNA in the pro-acinar/acinar cell compartment (the two cell types are indistinguishable at this resolution; results not shown). The ducts contained no cystatin S mRNA, but the

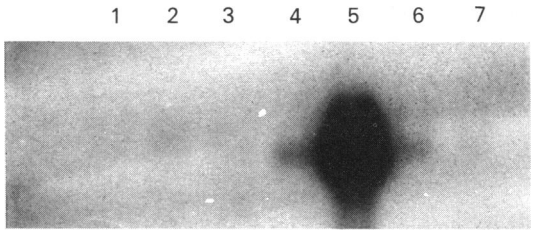

Fig. 3. Northern blot analysis of submandibular gland cystatin S mRNA in untreated female rats of different ages

Northern blot hybridization of total submandibular gland RNA (30 μg/lane) was carried out as decribed in the Materials and methods section. Lane 1, 20-day-old fetuses; lane 2, newborn; lane 3, 10-day-old animals; lane 4, 21-day-old animals; lane 5, 28-day-old animals; lane 6, 32-day-old animals; lane 7, 35-day-old animals.

Table 2. Effect of IPR on the weight of submandibular glands of developing female rats

Rats were injected with 0.1 μmol of IPR/g body wt. for 4 days and submandibular glands were removed 24 h after the last injection of the drug. The means and S.D. values are given; the number of animals included in each age group is given in parentheses.

Age (days)	Submandibular gland weight (g/100 g body wt.)	
	Control	IPR-treated
10	0.246 ± 0.028 (27)	0.350 ± 0.035 (23)
20	0.221 ± 0.018 (10)	0.441 ± 0.039 (10)
25	0.219 ± 0.015 (6)	0.401 ± 0.037 (6)
32	0.175 ± 0.014 (5)	0.289 ± 0.042 (5)

resolution was insufficient to exclude the presence of specific signals in the terminal tubule cells in the glands of 10–20-day-old animals.

DISCUSSION

Cystatins, cysteine proteinase inhibitors that belong to family 2 of the superfamily of cystatins (Barrett, 1986), are found in most biological fluids (Abrahamson *et al.*, 1986). Several forms of cystatins, which may result from the differential expression of cystatin genes, post-translational modification or heterogeneity in the *N*-terminal position of the polypeptides, have been isolated from human saliva (Isemura *et al.*, 1986). Although the structures of a number of cystatins and cystatin genes have been established, the regulation of cystatin genes in different cell types is largely unknown. As we have previously suggested (Shaw *et al.*, 1988; Shaw & Barka, 1989), the cystatin S gene in rat submandibular glands is regulated by β-adrenergic mechanisms, possibly via cyclic AMP. The β-adrenergic agonist IPR induced high levels of cystatin S mRNA in both adult and developing animals. Thus the rat cystatin S gene may be one of the

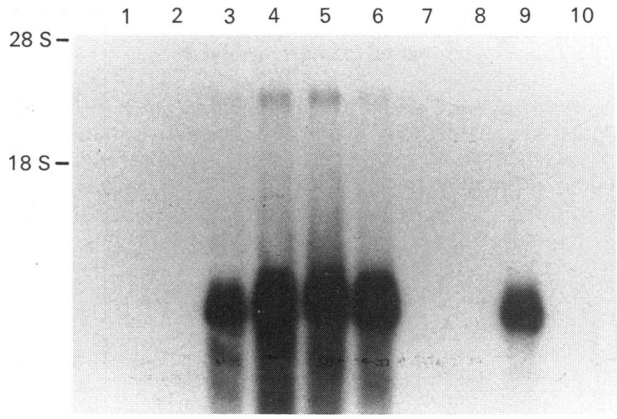

Fig. 4. Northern blot analysis of submandibular gland cystatin S mRNA from untreated and IPR-treated female rats of different ages

Northern blot hybridization of total submandibular gland RNA (20 μg/lane) was carried out as described in the Materials and methods section. Lane 1, 20-day-old fetuses; lane 2, newborn; lane 3, 10-day-old animals treated with IPR; lane 4, 20-day-old IPR-treated; lane 5, 25-day-old IPR-treated; lane 6, 32-day-old IPR-treated; lane 7, empty; lane 8, empty; lane 9, 1 μg of poly(A)$^+$ RNA from the submandibular glands of rats treated with IPR for 6 days, i.e. chronic drug treatment (a positive control); lane 10, empty.

cyclic AMP-regulated genes. Roesler *et al.* (1988) have suggested that the cyclic AMP-regulated genes fall into two categories: (1) genes which are rapidly regulated by cyclic AMP, and (2) genes whose transcription is increased only after several hours of cyclic AMP treatment. On the basis of these criteria, the rat cystatin S gene may belong to group 2, since the mRNA level did not increase until 4 h after the administration of IPR, a drug which is rapidly metabolized (Shaw & Barka, 1989). However, the rate of transcription, the half-life of cystatin S mRNA and its dependence, or lack thereof, on ongoing protein synthesis has yet to be established. Characterization of the rat cystatin S gene is also required as a prerequisite for the identification of cyclic AMP, hormonal and/or tissue-specific regulatory elements.

Our data suggest that there are several agents controlling expression of the rat cystatin S gene, including hormonal and tissue-specific factors. An example of tissue specificity is the high level of IPR-inducibility of cystatin S mRNA in the rat submandibular gland compared with the low level seen in the parotid gland (Shaw & Barka, 1989). Developmentally, cystatin S mRNA was detected in the submandibular glands of 21-day-old rats. The steady-state levels of the mRNA reached a maximum at 28 days of age. This was followed by a dramatic decrease to a barely detectable level at 32 days. Immunocytochemical studies (Yagil *et al.*, 1986) and hybridization studies *in situ* identified the pro-acinar and acinar cells as the main sites of synthesis of rat cystatin S mRNA and its corresponding protein. The postnatal increase observed in the concentration of steady-state cystatin S mRNA coincides with the differentiation of the acinar cells. The transiently high level of cystatin S mRNA in the glands of 4-week-old rats remains to be explained. Conceivably, it could be related to a transient reflex

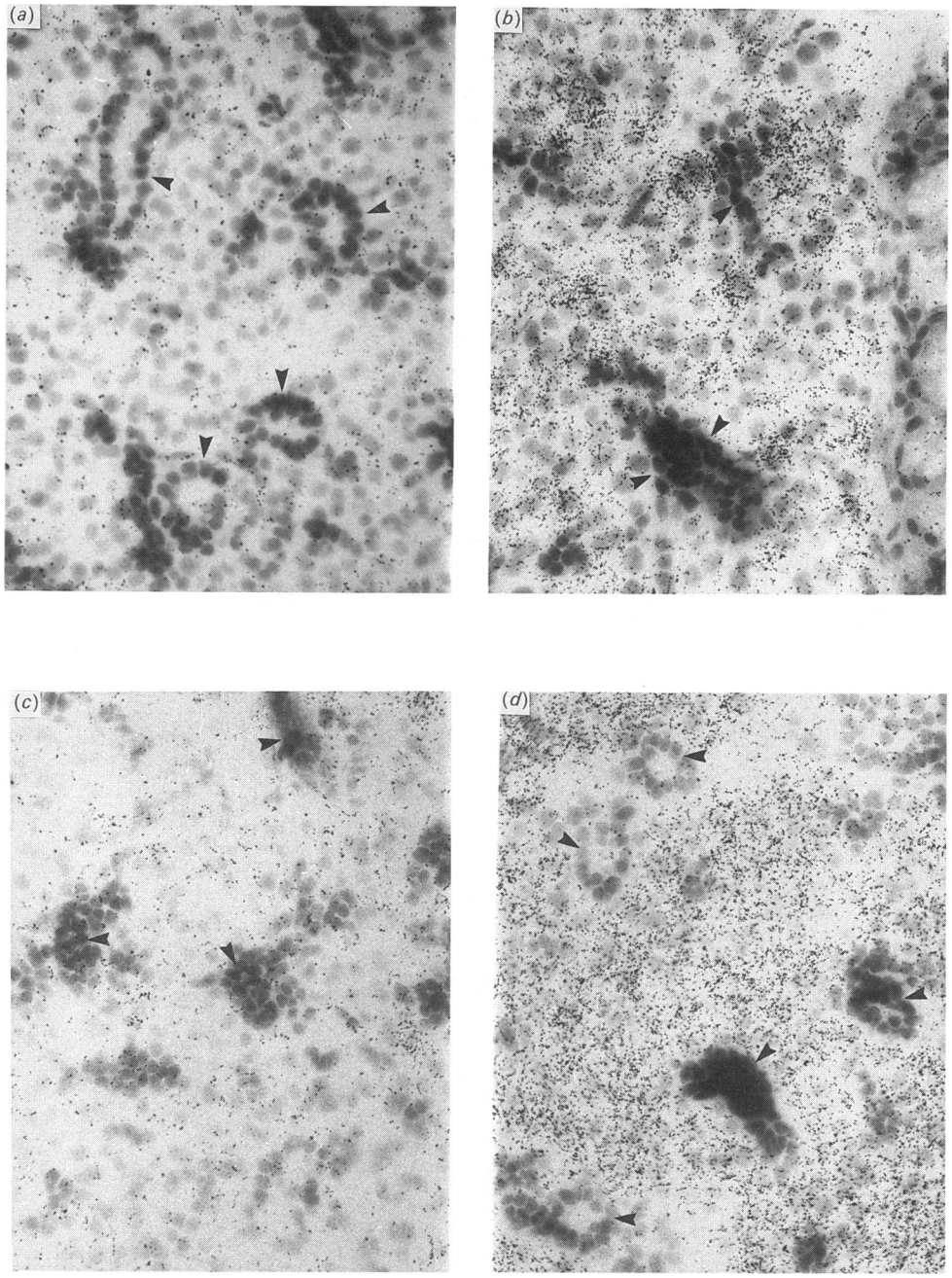

Fig. 5. Light micrographs of hybridizations *in situ* of cystatin S mRNA in sections of submandibular glands of adult rats

Preparation of sections and details of hybridizations *in situ* are described in the Materials and methods section. (*a*) Control male; (*b*) IPR-treated male; (*c*) control female; (*d*) IPR-treated female. Arrows indicate ducts. Magnification × 163.

stimulation of sympathetic outflow caused by an increase in mastication following the weaning of the animals. Such a stimulation, together with the long half-life of the message, could account for the repeatedly observed high concentration of cystatin S mRNA in the glands of rats of this age. It is known that an increase in mastication, e.g. by increasing bulk in the diet, causes a neurally mediated growth of salivary glands (Schneyer & Hall, 1976), which is presumably regulated through β-adrenoreceptors.

IPR induction of cystatin mRNA was more pronounced in the submandibular glands of female compared with male rats. The percentage of acinar cells in normally developing 10- and 15-day-old animals is the same, and, in addition, the percentage of acinar cells in female and male submandibular glands is the same (Alvares & Sesso, 1975). Therefore the difference in IPR-induced cystatin S mRNA in this age group is not a reflection of a higher proportion of acinar cells before administration of the drug, nor is it due to a difference in the percentage of acinar cells between the sexes. Furthermore, since this difference in inducibility was evident in 15-day-old animals, it is more than likely related to the sex genotype rather than to the level of circulating

steroid hormones. Another effect of IPR administration on salivary glands, stimulation of DNA synthesis, is also greater in female than in male rats (Barka, 1965).

Binding of catecholamines to β-adrenergic receptors results in the activation of adenylate cyclase and the intracellular formation of cyclic AMP, one of the most ubiquitous intracellular regulatory molecules (Levitzki, 1988). Cyclic AMP functions to co-ordinate diverse metabolic processes that, in the submandibular and parotid glands, include rapid changes in membrane permeability and ion fluxes, an increase in phospholipid turnover, an increase in intracellular Ca^{2+}, secretory discharge, and after a lag period, stimulation of DNA synthesis and growth and induction of gene expression. Whereas the metabolic effects of cyclic AMP on salivary glands have been extensively studied, progress in understanding the cyclic AMP induction of gene expression in these glands has been slow. So far, only the induction of the genes encoding proline-rich proteins, c-*fos*, 4β-galactosyltransferase and rat cystatin S has been documented. Whether IPR induction of these genes and the hyperplastic/hypertrophic effects of this drug are mechanistically related is not yet known. Although it is conceivable that cystatins, by inhibiting lysosomal cathepsins, are involved in the regulation of intracellular catabolism of proteins and hence in the mechanism of cellular hypertrophy, this idea remains solely conjectural at this time.

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