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β -ADRENERGIC EFFECTS ON SALIVARY GLANDS: GROWTH AND GENE REGULATION^{1,2}

TIBOR BARKA

*Departments of Cell Biology/Anatomy and Pathology, Mount Sinai School
of Medicine, New York, N. Y. 10029.*

In the parotid and submandibular glands β -adrenergic receptors play a pivotal role in regulating differentiation, cell replication and cellular and organ growth. Administration of the β -adrenergic agonist, isoproterenol, to rats or mice stimulates DNA synthesis and cell division of acinar cells, and given chronically, it produces marked hyperplastic/hypertrophic enlargements of these organs. The mechanisms by which IPR induces DNA synthesis and cellular hypertrophy are not known. A useful approach of analyzing these processes involves the identification of genes whose expression is regulated by β -adrenergic receptors. Several groups of genes regulated by adrenoreceptors in the salivary glands have now been identified. These include the proto-oncogenes *c-fos*, *c-myc*, *c-abl*, the family of proline-rich proteins, a cysteine proteinase inhibitor cystatin S, and 4 β -galactosyltransferase. Further studies are required to characterize genes which are positively or negatively regulated by β -adrenergic receptors in salivary glands, and to establish the relevance of the expression of these genes to control mechanisms of cell replication and growth.

The post-natal differentiation, growth, and functions of salivary glands are regulated by a complex interplay of hormones and the autonomic nervous system. In analyzing these regulatory functions, the salivary gland may be considered as consisting of four major compartments: 1. acinar, 2. intercalated duct, 3. granular convoluted tubule (GCT), and 4. striated duct—excretory duct. These compartment

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² Because of space limitation, only a limited number of references are cited in this publication.

differ not only in function, but also in response to regulatory inputs.

1. The production of primary saliva, isotonic with the plasma, is the primary function of the acinar cells. The major regulatory function on acinar cells is provided by the autonomic nervous system. Both parasympathetic and sympathetic nerves, via muscarinic cholinergic and adrenergic receptors, stimulate secretion of acinar cells, but differentiation and growth, and the expression of a number of genes are mostly regulated by β -adrenergic receptors. Some aspects of this regulation is the topic of this communication. Some hormones, notably estrogens, androgens, and thyroid hormones unquestionably affect the acinar cells, but their roles in regulating gene expression in these cells have not been elucidated.

2. Little can be said about the function(s) of intercalated duct cells which may serve as stem cells for both acinar and GCT cells during normal and hyperplastic growth. In some intercalated duct cells in rat submandibular gland secretory granules of unknown composition occur.

3. The GCT compartment, particularly in the gland of the mouse, is of great interest because the GCT cells synthesize and secrete a number of biologically active polypeptides (7). Long before the characterization of these polypeptides, the regulation of GCT cells by androgens, thyroid hormones, and to a lesser degree by corticosteroids and progesterone, was firmly established. The pivotal role of androgens in the differentiation and maintenance of GCT cells, that impart a sexual dimorphism on the gland, has been known since the nineteenforties. Following the characterization of genes encoding some of these polypeptides, notably epidermal growth factor (EGF), nerve growth factor (NGF), renin, kallikreins, and some of the peptidases, hormonal controls of GCT cells at the transcriptional level have been established. Notwithstanding some contrary reports, secretion of GCT cells seems to be mediated by α -adrenergic receptors.

4. Regulation of ion composition and water content of the saliva is the major function of the cells in the striated—excretory duct compartment. In addition, the striated duct cells have a limited secretory activity, and contribute to the very high level of kallikrein in the saliva. Hormones, notably the thyroid hormones, probably influence the functions of the striated duct cells, since they affect the expression of Na^+ , K^+ -ATPase in these cells (53).

Growth-promoting effects of β -adrenergic receptors on acinar cells.

The autonomic nervous system has a profound regulatory effect on the post-natal differentiation and growth of acinar cells in the parotid and submandibular glands of rats and mice, and probably of other species. This regulation is mediated primarily by β -adrenergic (β_1) receptors, and can be accentuated experimentally by the administration of the β -adrenergic agonist isoproterenol (IPR). As described first by Selye *et al.* (49), repeated administration of IPR produces conspicuous enlargements of the parotid and submandibular glands in rats. The same phenomenon was observed in mice (18). Subsequently, I have shown (4) that a single injection of IPR greatly stimulates DNA synthesis and cell replication in these glands, and suggested the IPR-stimulated salivary glands as a model to analyze the regulation of cell division *in vivo*.

The effects of IPR on the differentiation and growth of salivary glands have been studied extensively at the phenomenological level with few clues to the underlying mechanisms. The large volume of pertinent experimental data may be summarized

briefly as follows:

The rate of cell replication in the parotid and submandibular glands of adult rats and mice is very low, and it is responsible for the slow growth of these organs. Most acinar cells in these glands 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 of these glands with a lag period of 24–28 hr (4, 5, 16, 17). When the drug is given chronically, stimulation of DNA synthesis declines, but marked enlargements of the glands are produced (4, 18, 45, 49). The extent of this enlargement is indicated by the doubling of gland weight by two daily injections of IPR within 4–5 days. Results of a typical experiment, in which IPR was given daily for 2 weeks, are shown in Fig. 1. The enlargement, which is hyperplastic/hypertrophic in nature, is reversible. Even after prolonged administration of IPR, tumor development was never observed (48), and susceptibility to chemical carcinogens was not increased (8). Stimulation of DNA synthesis in the submandibular gland by IPR is more pronounced in female than in male rats (6), and it is age-dependent (9). During post-natal development the degree of stimulation 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 the drug causes an apparent acceleration of acinar cell differentiation (23, 46, 58). All these effects of IPR are

FIG. 1. The effect of daily administration of isoproterenol on rat submandibular gland. Adult female Sprague-Dawley rats were given 40 mg/kg of isoproterenol in doses for the number of 2 days indicated. The rats were given 0.5 μ Ci/g body weight of [^{3} H]thymidine 18 hr after the injection of IPR. One hour later the rats were exsanguinated under ether anesthesia, and DNA and incorporation of the labeled thymidine were measured by standard methods. There were 6–8 rats/group. Values are shown as percent of control which was taken as 100%. The control values were: gland weight: 312 ± 0.11 mg/100 g b. w.; DNA: 0.99 ± 0.17 mg/gland/100 g b. w.; cpm/mg DNA: $2.84 \pm 1.09 \times 10^3$. Bars indicate S. D. The differences between the control and experimental values were statistically significant ($p < 0.02$ or less), with the exception of DNA/gland in the 2-days group. The data are from ref. 4.

mediated by the adenylate cyclase-cAMP system and by calcium ions; administration of IPR leads to a rapid, transient increase in cAMP and intracellular Ca^{2+} in salivary glands.

IPR-cAMP regulates the expression of several genes in salivary glands.

Proto-oncogenes

That such profound effects of IPR on cellular differentiation and growth are exerted at the genetic level was already assumed in 1965 (5). Yet, it is only recently that studies on the regulation of gene expression by β -adrenoreceptors in salivary glands have been carried out, pioneered by Carlson and his co-workers dealing with the family of proline-rich proteins (PRP) (see below). Two such genes, whose expression in the salivary glands is regulated by β -adrenoreceptors and are studied in our laboratories, are the proto-oncogene *c-fos* and a cysteine proteinase inhibitor rat cystatin S.

The *c-fos* gene is the cellular homolog of the oncogene (*v-fos*) of the FBJ murine osteosarcoma virus (21). The *c-fos* gene is tightly regulated. Although the basal level of *c-fos* expression is very low in most cells, a great variety of agents can produce a dramatic but transient induction (20). On the basis of extensive experimental data, using mostly cultured cells, *c-fos* has been implicated in the regulation of cell replication and in specific differentiation pathways. Earlier works (reviewed in ref. 20, 36, 56) led us to the investigation of *c-fos* expression in the salivary glands in IPR-induced stimulation of cell replication and growth. We asked three specific questions: 1. Is *c-fos* expressed in the submandibular glands of developing or adult mice; 2. does the administration of IPR stimulate the expression of *c-fos* in the gland; and 3. does stimulation of *c-fos* expression correlate with stimulation of DNA synthesis and/or growth by IPR? In order to answer the third question, we have taken advantage of our previous findings that a single injection of IPR induces DNA synthesis and cell replication in the gland of adult animals but not in the glands of developing animals or in animals in which a hypertrophic/hyperplastic enlargement was produced by the chronic administration of the drug.

The experiments provided unequivocal answers to these three questions. 1. Neither in the glands of developing nor of adult mice was *c-fos* mRNA detected by Northern blot hybridization of total RNA. Hence, the basal level of *c-fos* expression in the mouse submandibular gland must be very low, below the level of detection by Northern blot hybridization. 2. A single injection of IPR caused a rapid, transient increase in *c-fos* mRNA levels in the glands of developing or adult mice, or in animals which had an IPR-induced enlargement of the gland. This stimulus also led to the accumulation of Fos protein in the acinar cells as demonstrated by immunocytochemical techniques. 3. There was no correlation between stimulation of DNA synthesis and the stimulation of expression of *c-fos* mRNA by IPR in the mouse submandibular gland. IPR induced *c-fos* expression even when it failed to stimulate DNA synthesis (10) (Table 1). These findings were supported by the data of Kousvelari *et al.* (32) who showed that IPR induces *c-fos* mRNA in parotid acinar cells also *in vitro*. In addition, these authors observed induction of *c-myc*, *c-cis*, and *c-abl* by IPR.

The role of *c-fos* in IPR-induced cell replication and growth remains unknown. Hypertrophic/hyperplastic enlargement *per se* does not involve an elevated level of *c-fos* mRNA or Fos protein, and the lack of correlation between stimulation of DNA syn-

TABLE 1. *The effects of isoproterenol on c-fos expression and DNA synthesis in the submandibular gland*

	c-FOS EXPRESSION	DNA SYNTHESIS
DEVELOPING ANIMALS	+++	—
ADULTS	+++	+++
CHRONIC IPR-TREATED	++++	—

Mice were given a single injection of 0.1 $\mu\text{mol/g}$ body weight of IPR. Northern blot hybridization of total RNA, extracted 30 min after the injection of the drug, was used to assess c-fos expression. DNA synthesis was assayed by measuring the incorporation of [^3H]thymidine into DNA 24 hr after the injection of IPR. Developing mice were 5 days of age. Animals in the chronic IPR-treated group were given 4 daily injections of IPR. The + signs indicate stimulation of c-fos expression or DNA synthesis compared to control animals. Details of the experiments and numerical values for the incorporation of [^3H]thymidine are given in ref. 10.

thesis and c-fos expression by IPR suggests that c-fos is either not in the regulatory pathway that leads from the membrane signal to cell replication, or it is insufficient to activate that pathway.

Subsequently, we and others have shown that β -adrenergic receptors induce c-fos expression in various cell types both *in vivo* and *in vitro*. These include the heart of the mouse, rat, and Syrian hamster, the brain of the mouse, the aorta of the rat, C6 glioma cells, and BC3H1 nonfusing muscle cells, (11, 12, 26, 59, and unpublished observations).

Several lines of evidence indicate that c-fos is a member of the class of inducible genes termed cellular immediate-early genes which also include c-jun. The protein products of these two genes, Fos and Jun, form a heterodimeric complex that associates with the nucleotide sequence motif known as the AP-1 binding sites. The Fos/Jun complex has been proposed to function as a transcriptional regulator that couples extracellular signals to alterations in cellular phenotype by regulating the expression of specific target genes (19, 22, 24, 25, 39, 41, 42, 47). So far, few such target genes have been identified (e.g. 54, 55), none in the salivary glands. Identification of such target genes in salivary glands is essential for revealing the cascade of cellular processes that leads to growth promotion by IPR-cAMP of the acinar cells.

Cystatins

Let me now turn to our unexpected findings concerning the adrenergic regulation of an other gene coding for a cysteine proteinase inhibitor, which we termed rat cystatin S, in the salivary glands of the rat.

It has been long known that the compositions of salivary glands and saliva are altered by the chronic administration of IPR. Among these alterations is the induction of a conspicuous protein that is essentially undetectable in the glands of untreated animals. This protein, given the operational name LM (large mobile) protein by Menaker *et al.* (34), was purified and partially characterized by Naito (37, 38) as a 13,000 molecular weight polypeptide of unknown function. We have focused our at-

tention on this protein because it appeared to be unique in the submandibular glands and saliva of IPR-treated rats.

Immunocytochemical studies revealed that the glands of IPR-treated young rats contained high concentrations of the LM protein localized primarily in the acinar cells, while the protein was undetectable in the glands of untreated animals (57). Subsequently, we have also shown, using a rabbit reticulocyte lysate *in vitro* translation system, that the glands of IPR-treated rats contained relatively high concentrations of mRNA coding for the LM protein in contrast to the glands of untreated animals (13). With the availability of antibodies against the LM protein, and using poly (A)⁺ RNA from the glands of 11-day old rats treated with IPR for 4 days, which is greatly enriched in LM mRNA, we have cloned and sequenced cDNAs encoding the LM protein (52). One of these clones contained the complete nucleotide sequence encoding the mature LM protein consisting of 118 amino acids, part of a signal sequence, and a 226-nucleotide long 3'-noncoding sequence. Using the putative amino acid sequence deduced from the nucleotide sequence we have searched protein data bases for sequence similarities with proteins of known sequences. The computer search revealed that the LM protein has a high degree of sequence similarity to a number of cysteine proteinase inhibitors called cystatins. These sequence similarities suggested that the LM protein is a cysteine proteinase inhibitor, and this was subsequently proven by enzyme inhibitor assays. The LM protein, purified from the saliva or the submandibular glands of IPR-treated rats strongly inhibited papain and ficin, but not bovine cathepsin B. On the basis of these data, and keeping with the recommended nomenclature (14), we termed the LM protein rat cystatin S, a new member of the cystatin family 2 of the cystatin superfamily.

Rat cystatin S mRNA was undetectable in the submandibular or parotid glands of adult rats by Northern blot hybridization of total RNA. However, high levels of the message were present in the glands of rats treated with daily injections of IPR for 4-6 days. These elevated levels of cystatin S mRNA persisted for several days after discontinuation of the IPR treatment, suggesting that the half-life of the message is relatively long. A single injection of IPR also induced cystatin mRNA as early as 4 hr. Again, high levels of the mRNA were demonstrable even 72 hr after a single injection of the drug. Induction of cystatin mRNA by IPR was more pronounced in female than in male animals, just as stimulation of DNA synthesis is also more pronounced in female rats compared to that of males. This sexual difference in inducibility was observed also in pre-pubertal rats, suggesting that the difference is more likely related to the sex genotype than to the level of circulating sex hormones. In general, the level of mRNA in the submandibular gland was about 20-fold higher than in the parotid gland (50).

Cystatin S mRNA was not detected in the submandibular glands of 20-day old fetuses, or in the glands of rats up to three weeks of age. However, after this time cystatin mRNA levels increased reaching a peak around four weeks of age. It then declined rapidly to a barely detectable level in the glands of 32 days old animals. During the entire post-natal development period administration of IPR for 4 days led to high levels of cystatin mRNA. *In situ* hybridization revealed cystatin mRNA only in the proacinar and acinar, but not in the duct cells. (51).

Cysteine proteinases, one of the major classes of proteinases in mammalian cells which include the lysosomal cathepsins B, L, N, S, M, and T, and several

multicatalytic and metal-dependent proteinases are involved with intracellular degradation of proteins. Extracellularly, when released or secreted by cells or bacteria, these enzymes can cause cell injury, and, in concert with other proteolytic enzymes, are responsible for tissue degradation under various pathologic conditions. The activities of cysteine proteinases are controlled by naturally occurring inhibitory proteins, cystatins. Isolation and characterization of cystatins of diverse origin led to the recognition that all known cystatins are members of a cystatin superfamily consisting of the 1. stefin, 2. cystatin, and 3. kininogen families (15). Members of the cystatin family, which include chicken cystatin, human cystatins C, S, SN, SA-1, and rat cystatin S, occur both intracellularly and extracellularly in various tissue fluids such as saliva, tears, cerebro-spinal fluid, semen (1, 15, 30, 31).

Our findings are the first to show the regulation of the cystatin gene in the salivary glands by β -adrenergic receptors, and raises the question of the function(s) of this inhibitor in the salivary glands. Human cystatins S, SN, SA-1, and several other forms of cystatins are excreted into the saliva. Rat cystatin S is also found in high concentrations in the saliva of rats treated with IPR. Cystatins in saliva are presumed to provide protection against the harmful effects of cysteine proteinases of cellular or bacterial origin. Whether rat salivary glands contain also cystatins which function intracellularly is not known. Since the accumulation of cystatin in the submandibular and parotid glands parallels the hypertrophic growth produced by the chronic administration of IPR, it is tempting to speculate that this protein causally contributes to the production of cellular enlargement by inhibiting lysosomal proteinase responsible for intracellular degradation, and hence, the turnover of proteins.

Proline-rich proteins

The first group of proteins whose induction by β -adrenergic receptors in salivary glands of several species was discovered is the family of proline-rich proteins (2, 3, 33, 35). It is outside of the scope of this review to discuss the structure and regulation of the multigene families encoding the various forms of PRPs. Since these proteins accumulate also in high concentrations in the parotid glands of rats or mice enlarged by the chronic administration of IPR, they may contribute to cellular hypertrophy.

4 β -Galactosyltransferase

Induction of 4 β -galactosyltransferase (GT) in rat parotid gland by IPR is an other example of regulation of gene expression by adrenoreceptors in salivary glands. Humphreys-Beher and his co-workers have shown that the IPR-induced hypertrophy of the parotid gland is accompanied by an increase in GT in the plasma membranes, and it results in secretion of the enzyme into the saliva. The increase in GT activity is caused by an increase in gene expression. The findings that the IPR-induced hypertrophy of the parotid gland is blocked by the GT modifier protein α -lactalbumin and by antisera to bovine GT are of particular interest and relevant to the mechanism of hypertrophy induced by the drug (27-29).

Perspectives

cAMP is probably the most ubiquitous regulatory molecule which exerts pleiotropic effects in coordinating diverse metabolic processes. In the submandibular and parotid glands, the growth-promoting effects of β -adrenergic receptors are mediated by the adenylate cyclase-cAMP system. In recent years, a number of cAMP-regulated genes have been isolated and characterized. In such genes, two

classes of cis-acting elements have been identified (43). Genes whose transcriptions are rapidly altered in response to cAMP (e. g. P-enolpyruvate carboxykinase, somatostatin, vasoactive intestinal polypeptide, c-fos) carry a highly conserved 8-bp palindromic sequence [T(G/T)ACGTCA], originally termed CRE (c-AMP regulatory element). Recently, a different cAMP-responsive element was identified, and it is referred to as the Activator Protein 2 (AP-2) binding site. Genes having the AP-2 element (e. g. human metallothionein II_A, human growth hormone, rat prolactin) also respond by a rapid change in the rate of transcription to cAMP. The cAMP responsive elements in rat cystatin S gene, in human cystatin genes, in the PRP genes, and the GT gene have not been identified or mapped. However, the human salivary cystatin S gene contains an 8-bp element (−47 GCTGGAGG −40) with considerable similarity to some of the documented CRE elements (44).

As discussed above, the number of known genes whose expression in the salivary glands is regulated by cAMP is very small. However, a rapid progress in this field can be expected. Equally important is to identify those genes which are negatively regulated by cAMP in these glands. In the salivary glands IPR stimulates cell replication and hypertrophic growth. These two processes can be discerned operationally, and must have different mechanisms. For one, stimulation of cell replication is an 'all or none' phenomenon triggered by a single external signal, and probably it depends on an intracellular regulatory factor reaching a critical level. Such a putative factor may play a positive or negative role in triggering G₀ cells to enter into the cycle. In contrast, production of cellular and organ hypertrophy requires sustained or repeated exposures to the drug or condition, and it appears to be a continuous process. During the past decade, considerable progress has been made concerning the kinetics of cell cycle and the regulation of cell proliferation based on *in vitro* studies using primarily fibroblasts (40). The problems of analyzing the mechanisms of cell cycle regulation and cellular hypertrophy *in vivo* are more formidable. For such studies, the IPR-stimulated salivary gland remains a useful experimental system. Applications of molecular biological approaches will contribute to the task of establishing sequential, causal relationships among the multiple alterations of gene expression during this *in vivo* model of cell replication and growth.

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