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Induction of Proline-rich Proteins in Hamster Salivary Glands by Isoproterenol Treatment and an Unusual Growth Inhibition by Tannins*

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Treatment of hamsters with the β-agonist isoproterenol caused a dramatic increase in a series of unusual proteins in the parotid and submandibular glands. These proteins are acid soluble and they contain high amounts (mol %) of glutamate plus glutamine (30–35), proline (23–30), and glycine (12–25). Three proteins (HP45, HP43a, and HP43b) were isolated from trichloroacetic acid extracts of parotid glands of isoproterenol-treated hamsters. The basic protein (HP45) was not retained by DEAE-cellulose and did not contain phosphate or carbohydrate. Two acidic proteins (HP43a and HP43b) had the same apparent molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, but these were separated by DEAE-cellulose chromatography. HP43a and HP43b contained 4.3 and 5.7 phosphate residues/mol of protein, respectively. Levels of mRNAs encoding this series of proteins showed striking increases following isoproterenol treatment as determined by cell-free translations and Northern analysis. Feeding tannins to rats and mice mimicks the effects of isoproterenol treatment on the parotid gland (Mehansho, H., Hagerman, A., Clements, S., Butler, L., Rogler, J., and Carlson, D. M. (1983) *Proc. Natl. Acad. Sci. U. S. A.* 80, 3948–3952; Mehansho, H., Clements, S., Sheares, B. T., Smith, S., and Carlson, D. M. (1985) *J. Biol. Chem.* 260, 4418–4423)). However, hamsters on a high tannin diet (2%) did not respond like rats and mice and instead displayed an unusual growth inhibition. Weanling hamsters maintained on a 2% tannin diet initially lost weight for 3 days and then failed to gain weight for up to 6 months when kept on this diet. Essentially a normal growth rate was observed when the tannin-fed hamsters were switched to a normal diet.

Salivary glands of various animals can synthesize, or can be induced to synthesize, a group of proteins which are unusually high in proline, the so-called proline-rich proteins (PRPs)[1] (1). These proteins collectively constitute about 70% of the proteins in human salivary secretions (2). The PRPs are encoded by multigene families (1) and can undergo various post-translational modifications including proteolysis, phosphorylation, and glycosylation. These unusual proteins are presumably constitutive in human saliva (2), but families of similar proteins are dramatically increased or induced in parotid and submandibular glands of rats (3, 4), mice (5, 6), and hamsters (this report) by isoproterenol treatment. The nucleotide sequences of several PRP mRNAs from rat (7, 8), mouse (8), and human (9) and the structures and organizations of complete genes of PRP multigene families from the mouse (10), hamster (11), and human (12) have been reported. Proteins derived from the nucleotide sequences are all characterized by four general regions: a putative signal peptide, a transition region, the repetitive region, and a carboxyl-terminal region (1).

Previously we found that feeding tannins mimicked the effects of isoproterenol on parotid glands, causing glandular hypertrophy and induction of PRPs in rats (5) and mice (13). The apparent tissue-specific synthesis and the appearance of PRPs in saliva suggest a biological function in the oral cavity and gastrointestinal tract. Evidence has been presented that these proteins have high affinities for tannins and that they can reverse the detrimental effects of tannins in the diets of rats (13) and mice (5). However, hamsters do not respond to dietary tannins by inducing the synthesis of PRPs and as a result tannins show an unusual ability to inhibit growth. Tannins are also unusually toxic to hamsters. We have isolated and partially characterized three proteins from hamster parotid glands which are high in glutamate (or glutamine), proline, and glycine and which are dramatically induced by isoproterenol treatment. In an attempt to determine the reasons for the different responses to tannins, assays for β-adrenergic receptors and adenylate cyclase were performed on membrane preparations from parotid glands of rats and hamsters. Cell-free translations and Northern analysis were carried out on control and isoproterenol-treated animals to determine the extent of induction of PRP mRNAs.

EXPERIMENTAL PROCEDURES

Materials—All materials were of highest purity available and were purchased from commercial sources unless otherwise indicated. The following were purchased from respective companies: L-[3,4-^{3}H] proline (100 Ci/mmol), gelatin, ICN or Nutritional Biochemicals; 3-[^{125}I] iodocyanopindolol (200 Ci/mmol), Amersham Corp.; *dl*-isoproterenol·HCl, ATP, cAMP, lysozyme, phosphoenolpyruvate, pyruvate kinase, binding protein, chymotrypsin, Sigma; EN^{3}HANCE, Du Pont-

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[1] The abbreviations used are: PRP, proline-rich protein; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; H29, hamster proline-rich protein gene; CNBr, cyanogen bromide.

New England Nuclear; molecular weight standards, Bio-Rad; trypsin-tosylphenylalanyl chloromethyl ketone, clostripain, Worthington; *Staphylococcus aureus* protease, Miles Laboratories Inc.; Quebracho extract, Frask Chemical Co.; reticulocyte lysate, gift from Dr. David Kuhn, Department of Biochemistry, Purdue University; high tannin sorghum (Savanna), and low tannin sorghum (RS-610) were grown at the Purdue University Agronomy Farm.

Feeding Trials—Male Golden Syrian hamsters (50-60 g) were maintained on Purina Lab Chow for 5-6 days before starting the feeding experiments. Sorghum diets were prepared as described elsewhere (13). Feed and water were provided *ad libitum*. Tannin contents were measured by a competitive binding assay (14). The condensed tannin contents of Savanna sorghum grain and Quebracho extract were 2 and 50%, respectively. Gelatin, Quebracho extract, or an amino acid mix equivalent to gelatin in amino acid composition was added to sorghum diets as indicated at 4% of the dry weight.

Isoproterenol Treatment—Male Golden Syrian hamsters (90-100 g) fed Purina Lab Chow were utilized as gland donors. Each hamster was injected intraperitoneally with the indicated amount of *dl*-isoproterenol·HCl daily as described by Muenzer *et al.* (3). Unless otherwise indicated, hamsters were treated daily with 1.0 mg of isoproterenol for 10 days.

Isolation of Proline-rich Proteins—Animals were anesthetized with sodium pentobarbital and killed by exsanguination. The parotid and submandibular glands were removed and stripped of connective tissue and fat. Trichloroacetic acid extractions were performed as described by Mehansho and Carlson (4). The acid-soluble fraction, which contains most of the proline-rich proteins, was further fractionated by DEAE-cellulose chromatography. About 2.0 mg of sample was applied to a column (1.5 × 70 cm) which had previously been equilibrated with 5 mM phosphate buffer, pH 8.0. After washing with 150 ml of the equilibrating buffer, elution was carried out with a linear gradient ranging from 0 to 1 M NaCl.

Membrane Preparation—Crude membranes were prepared as described by Ludford and Talamo (15) with a slight modification. Fresh parotid glands from normal rats and hamsters were homogenized in 20 volumes (g/ml) of buffer A (12.5 mM sodium phosphate, pH 7.4, 0.3 M NaCl, and phenylmethysulfonyl fluoride (15 μg/ml)) for 30 s, and the extract was centrifuged at 31,000 × *g* for 1 h. One-half of the pellets were suspended in the same volume of Buffer A (20 ml/g tissue) with a Dounce homogenizer. This crude membrane preparation was used for the ^{125}I-hydroxybenzylpindolol binding experiments. The remainder of the pellets was suspended in 4 volumes of cAMP assay buffer (50 mM Tris·HCl, pH 7.5, 4 mM EDTA), and this membrane preparation was used for assaying adenylate cyclase activity.

β-Adrenergic Receptor Binding Assay—The ability of the membrane preparations to bind a β-agonist was determined by using ^{125}I-hydroxybenzylpindolol (15). A final volume of 1 ml contained 0.5 ml of membrane suspension, 0.2 mg of lysozyme, 10 mM GlcNAc, and 200 pmol of ^{125}I-hydroxybenzylpindolol. The assay mixture was incubated at 37 °C for 1 h. Assays were carried out in the presence and absence of 10 μM *dl*-propranolol. Membranes were collected on glass-fiber filters, and bound ^{125}I-hydroxybenzylpindolol was measured in a γ counter.

Adenylate Cyclase Assay—Adenylate cyclase activities of parotid membrane preparations were measured according to the procedures of Ludford and Talamo (16) and Tovey *et al.* (17). Assay mixtures (final volume, 100 μl) consisted of 40 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM theophylline, 0.1 mM EDTA, 10 mM phosphoenolpyruvate, 25 μg of pyruvate kinase, 1 mM ATP, and 50 μl of membrane suspension. These were incubated for 10 min at 37 °C. Amounts of cAMP generated were estimated by the competitive protein binding technique (17).

Protein Determination—Proline-rich proteins usually lack aromatic amino acids and are estimated by absorbance at 230 nm. The calculated extinction coefficient for one of these proteins is $E^{1\%}_{1cm}$ = 25 (4). Protein in membrane preparations was measured by the Lowry method (18) using bovine serum albumin as a standard.

Amino Acid Analysis—Protein samples (0.1-0.3 mg) were hydrolyzed and analyzed as described earlier (4).

Protein Sequence Analysis—The amino-terminal regions of three proteins from hamster parotid glands (HP45, HP43a, and HP43b) were sequenced according to Hermodson *et al.* (19).

Proteolytic Digestions of HP45 and HP43a—Incubation conditions for protease treatments were: clostripain, 50 mM ammonium bicarbonate, 0.27 M mercaptoethanol, 37 °C, 4 h; *S. aureus* protease, 50 mM ammonium acetate, 37 °C, 18 h; trypsin, 0.01 M HCl, 37 °C, 6 h; chymotrypsin, 0.5% $NaHCO_3$, 37 °C, 6 h. Enzyme/substrate ratios (w/w) for clostripain, *S. aureus* protease, trypsin, and chymotrypsin were 1:50, 1:40, 1:50, and 1:50, respectively. Cyanogen bromide digestion was performed as described (20).

Isolation and Analysis of mRNA—Total RNA was prepared by using the guanidine thiocyanate-cesium chloride procedure described in Chirgwin *et al.* (21). RNA was translated in a rabbit reticulocyte lysate system, and the translation products were analyzed as described previously (6). Northern blot analysis was performed as described by Thomas (22) using electrophoresis in 1.5% agarose gel containing 2.2 mM formaldehyde (23). ^{32}P-Labeled exon III of hamster PRP gene H29 (11) was prepared by nick translation and was used as a probe for hybridizations. The filters were treated as described previously (6).

RESULTS

Effects of Isoproterenol Treatment—Isoproterenol treatment has a dramatic hypertrophic effect on the parotid and submandibular glands of rats (3, 4) and mice (5) with the sizes of these glands increasing by about 10- and 5-fold, respectively, after 10 days of treatment. Increases in sizes of the parotid and submandibular glands of hamsters were essentially negligible (≤0.5-fold), but isoproterenol treatment of hamsters did induce the synthesis or accumulation of acid-soluble proteins about 6-8-fold (Table I). As found with rats (3) and mice (5), these proteins were high in proline but were also unusually high in glutamate (or glutamine) (Table II).

Electrophoretic patterns of acid-soluble proteins and glycoproteins induced in salivary glands of hamsters treated with

TABLE I

Effects of isoproterenol treatment on gland weights and on amounts of acid-soluble proteins

	Gland weight		Acid-soluble proteins	
	Parotid	Submandibular	Parotid	Submandibular
	g		*mg/g tissue*	
Isoproterenol				
0	0.32	0.70	5.5	10.7
0.5 mg	0.42	0.80	22.8	48.1
1.0 mg	0.38	0.85	29.7	82.6
1.5 mg	0.40	0.80	27.6	63.5
2.5 mg	0.50	1.00	37.9	67.4
3.5 mg	0.50	0.85	31.6	83.5

TABLE II

Amino acid compositions

	HP45	HP43a	HP43b	H29[a]
	mol/100 mol			
Asx[b]	7.9	10.3	10.8	10.7
Thr	0.1	1.4	1.6	1.2
Ser	0.6	3.7	4.2	4.7
Glx[c]	31.7	34.1	30.7	34.9
Pro	30.8	22.6	23.5	20.1
Gly	24.8	12.2	14.1	14.2
Ala	ND[d]	1.9	2.3	1.7
Ile	ND	0.5	0.8	1.2
Leu	0.5	1.3	1.4	1.8
Tyr	ND	ND	0.6	0.6
His	0.5	3.0	3.1	1.2
Lys	1.3	5.1	3.4	3.6
Arg	0.9	3.9	3.3	3.6
Met	ND	ND	0.2	0
Phe	0.9	ND	ND	0.6
mol of PO_4^{3-}/mol of protein	0	4.3	5.7	

[a] Amino acid composition derived from the nucleic acid sequence of hamster PRP gene H29 (11) as given in Fig. 9 and assuming ^{15}Ala is the N-terminal amino acid of the mature protein.

[b] Asx = Asp + Asn.

[c] Glx = Glu + Gln.

[d] ND, not detected.

varying amounts of isoproterenol are shown in Fig. 1. The profiles and levels of most proteins were essentially unchanged when the amount of isoproterenol was increased from 0.5 to 3.5 mg/animal. Several proteins are glycosylated, especially in isoproterenol-treated submandibular glands (Fig. 1*B*). A glycoprotein of $M_{r(app)}$ of 89,000 (GP89) showed an unusual regulatory pattern; it was induced dramatically in parotid glands of hamsters treated with 2.5 mg of isoproterenol/day.

Protein Isolations—The acid-soluble proteins extracted with 10% trichloroacetic acid from parotid glands of isoproterenol-treated hamsters were partially resolved by DEAE-cellulose chromatography (Fig. 2). Fractions in peaks I, III, IV, and V lacked absorbance at 280 nm, which is consistent with results obtained with other PRPs (3). To check for homogeneity, each fraction from peaks I through V was assayed by SDS-PAGE (data not shown). As illustrated in Fig. 2 (*inset*), peak I which was not retained by the DEAE-cellulose column appears as one protein in SDS-PAGE, and this protein has a $M_{r(app)}$ of 45,000 (HP45). Peak III includes at least 3 proteins. Peaks IV and V contain glycoproteins HP43a and HP43b, each with a $M_{r(app)}$ of 43,000. A minor contaminant of HP43b was removed by repeating the DEAE-cellulose chromatography with a lower salt gradient.

Compositional Analysis—The amino acid compositions of HP45, HP43a, and HP43b are presented in Table II. These proteins are all high in glutamic acid (or glutamine), proline, glycine, and aspartic acid (or asparagine). They either lack or contain very low amounts of aromatic and sulfur-containing amino acids. HP43a and HP43b are different, but they do have similar amino acid compositions. HP43a and HP43b contain 4.3 and 5.7 mol of phosphate/mol of protein, respectively (Table II). The amino acid composition of the protein encoded by hamster PRP gene H29 (11) is similar to, but not identical with, either HP43a or HP43b.

Partial Amino Acid Sequences of HP45, HP43a, and HP43b—Sequences of the amino-terminal regions of HP45, HP43a, and HP43b are compared together with sequence data from proline-rich proteins of rat, mouse, and human (Fig. 3). There is about 73% homology in the first 11 amino acids of HP43a and HP43b. When the sequence of HP43a is compared with the amino acid sequence derived from PRP gene H29 (residues 15–25) (11), there is a single substitution at position 8; aspartic acid (HP43a) (codon GA$^{T}/_{C}$) for isoleucine (H29) (codon ATA). When sequences of HP43a and HP43b were aligned with peptides encoded by PRP cDNAs of mouse (pMP125) and rat (pRP33), there were two regions of homology for all four peptides. The basic PRP, HP45, had no homology with either HP43a or HP43b (or with other basic PRPs from rat (8), mouse (8), and human (24)). Both HP43a and HP43b have relatively high amounts of serine and threonine which are potential sites for phosphorylation and glycosylation. For example, residue 9 in HP43a and HP43b is missing from sequence analysis as would be expected if it was either glycosylated or phosphorylated.

Treatment with Proteases and CNBr—HP45 and HP43a were treated with various proteases and with CNBr. A characteristic difference was observed between HP45 and HP43a (Fig. 4). HP45, the basic PRP, was resistant to *S. aureus* protease V8 whereas HP43a was an excellent substrate. Both behaved similarly toward clostripain and trypsin. Chymotrypsin hydrolyzed HP45 extensively, but HP43 appeared to have a single cleavage site. As expected, both HP45 and HP43a were resistant to CNBr.

Binding Assays of Hamster Proline-rich Proteins to Tannins—A high affinity for tannins is one of the characteristic properties of PRPs (13). The relative affinities of tannins for four PRPs from hamster parotid glands were measured by a binding assay using ^{14}C labeled bovine serum albumin (14). These proteins have about an 8-fold greater affinity for tannin than does bovine serum albumin (Fig. 5). Results of these binding assays are comparable to those obtained for rat (13) and mouse (5) PRPs.

Cell-free Translations—Total RNAs prepared from parotid glands of isoproterenol-treated and control hamsters were translated *in vitro* by the reticulocyte lysate system (Fig. 6) in the presence of [^{3}H]proline. Isoproterenol treatment increased demonstrably the *in vitro* synthesis of several proteins. Many proteins induced by isoproterenol treatment were apparently present normally in low amounts. Two polypeptides (M_r 58,000 and a low molecular weight protein) disappeared with isoproterenol treatment. This observation is consistent with the reduction or disappearance of α-amylase and parotid-specific protein in cell-free translations of RNAs from isoproterenol-treated rats (7) and mice (6). Cell-free translations with [^{35}S]methionine (not shown) clearly demonstrated the dramatic decrease in α-amylase.

Northern Hybridization—Relative changes of PRP mRNAs after isoproterenol treatment were determined by Northern analysis using exon III of hamster PRP gene H29 (11) as the probe (Fig. 7). Isoproterenol treatment caused about a 10-fold induction in PRP mRNAs. This increase is considerably lower

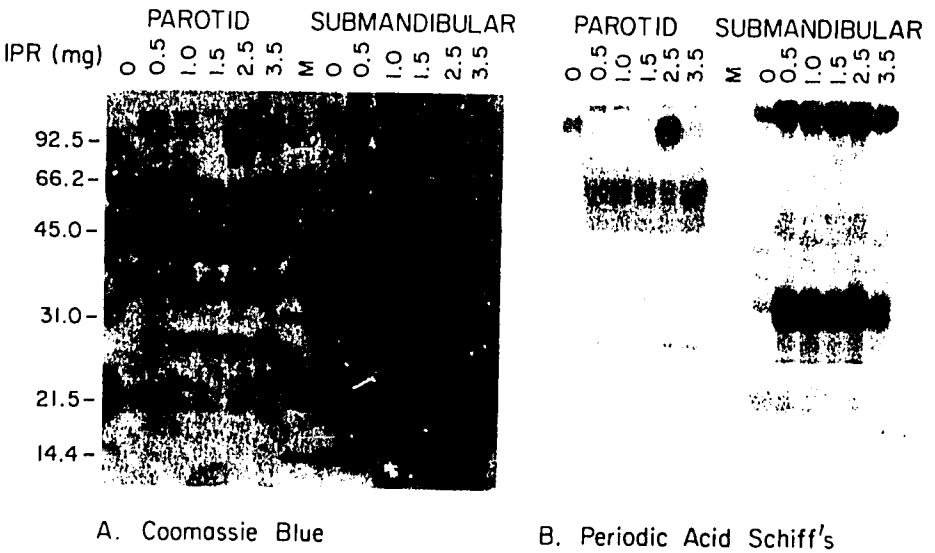

FIG. 1. **SDS-polyacrylamide gel electrophoresis of acid-soluble fractions from the parotid and submandibular glands of control and isoproterenol (*IPR*)-treated hamsters.** Acid-soluble extracts equivalent to 3 mg of tissue were applied to each lane. *A*, protein staining; *B*, carbohydrate staining; *M*, molecular mass markers in kilodaltons; phosphorylase *b*, 92.5; bovine serum albumin, 66.2; ovalbumin, 45; carbonic anhydrase, 31; soybean trypsin inhibitor, 21.5; and lysozyme, 14.4.

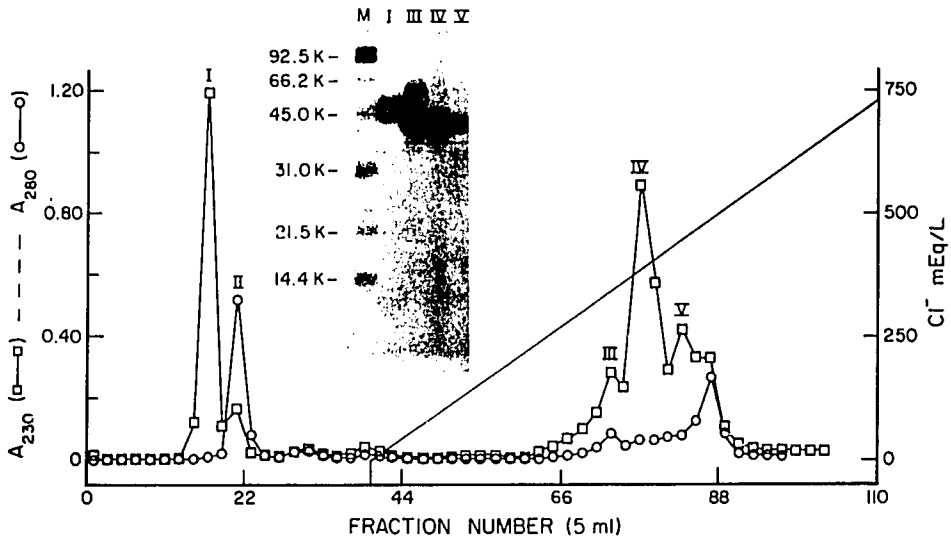

FIG. 2. **DEAE-cellulose chromatography of the acid-soluble components from hamster parotid glands.** Elution was performed as described under "Experimental Procedures." Analysis of peaks, I, III, IV, and V by SDS-polyacrylamide gel electrophoresis is shown in the *inset*.

Hamster HP43b:	Ala - Thr - Leu - Tyr - Glu - Asp - Ser - Pro - XXX - Ser - Leu -
Hamster HP43a:	Ala - Thr - Ile - Tyr - Glu - Asp - Ser - Asp - XXX - Gln - Leu -
Hamster H29:	Ala - Thr - Ile - Tyr - Glu - Asp - Ser - Ile - Ser - Gln - Leu -
Rat pPP33:	Glu - Val - Ile - Tyr - Glu - Asp - Ser - Ser - Ser - Gln - Leu -
Mouse pMP125:	Glu - Ile - Thr - Tyr - Glu - Asp - Ser - Asp - Ser - Gln - Leu -
Human Protein A:	Glu - Asp - Leu - Asn - Glu - Asp - Val - Ser - Gln - Glu - Asp -
Human IB-1:	Glu - Asn - Leu - Asn - Glu - Asp - Val - Ser - Gln - Glu - Glu
Hamster HP45:	Phe - Pro - Gln - Gly - His - Pro - Asn - Gln - Gly - Phe - Gly - Pro - Lys - Gln -

FIG. 3. **Comparison of the N-terminal regions.** H29, hamster PRP gene (derived sequence) (11); HP45, hamster basic PRP; HP43a and HP43b, hamster acidic PRPs (in this report); pRP33, rat cDNA encoding an acidic PRP (7); pMP125, mouse cDNA encoding acidic PRP (8); protein A, human acidic PRP (2); IB-1, human basic PRP (24); *XXX* are likely glycosylated or phosphorylated serines or threonines.

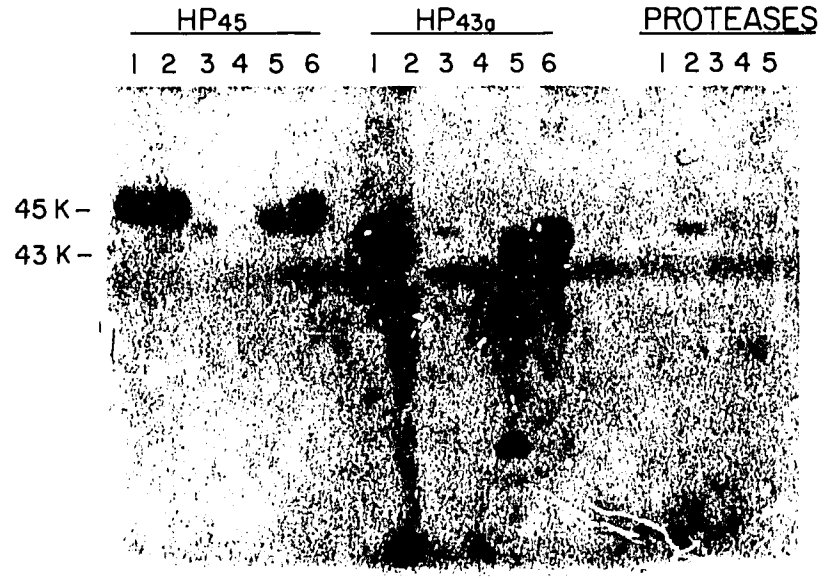

FIG. 4. **Protease and CNBr treatment of HP45 and HP43a.** *1*, no enzyme; *2*, *S. aureus* V8; *3*, clostripain; *4*, trypsin; *5*, chymotrypsin; *6*, CNBr. Protease digestions and CNBr cleavage were performed as described under "Experimental Procedures." Molecular weight markers are indicated by *dashes*.

than the 70-fold increase observed in mice (6). Parotid glands of hamsters, however, have higher basal levels of PRP mRNAs than did the mouse. As observed in rat (8) and mouse (6), two major size classes of RNAs were detected.

Effects of Feeding a Diet High in Tannin—Unlike with rats (13) and mice (5), feeding high tannin sorghum (Savanna) to hamsters for 3 days failed to cause either glandular hypertrophy or an increase in the levels of PRPs (Table III). Upon prolonged feeding of high tannin sorghum, an unusual inhibition of growth was observed (Fig. 8). In fact, after 6 months on Savanna sorghum, hamsters were essentially (±5 g) the same weight as at 3 days (data not shown). At this point (6 months), diets were switched and hamsters previously on Savanna grew at close to the normal rate observed for the younger animals. Hamsters switched to the high tannin diet lost about 20% of their weight and within about 20 days after the diets were switched, both groups were close to the same weight. Growth curves for rats on high and low tannin diets are shown in Fig. 8 (*inset*). The addition of either gelatin or the amino acid mix to the low tannin diet had little effect on growth rate (Table IV). In contrast, when gelatin, which also has a high affinity for tannins, was added to the high tannin diet, the weight gains of the hamsters improved dramatically (Table IV) and were essentially the same as hamsters maintained on low tannin sorghum. Savanna plus an amino acid mix equivalent to the composition of gelatin, on the other hand, had no effect on the tannin-mediated growth inhibition. The lack of effect of the crystalline amino acid mix eliminates the possibility that the beneficial response to gelatin in the high tannin diet involves the amino acids or total nitrogen

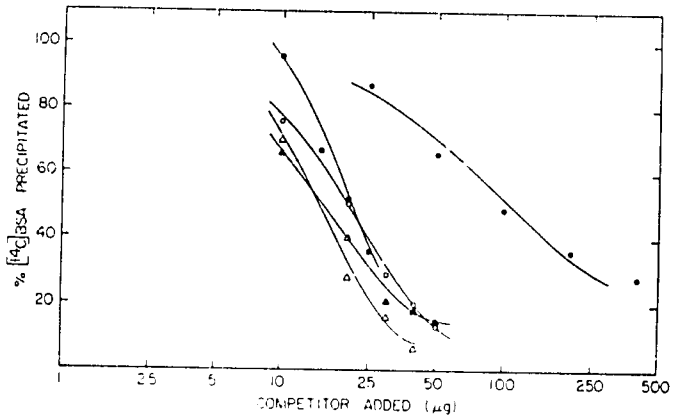

FIG. 5. **Competitive binding curves between ^{14}C-labeled bovine serum albumin (*BSA*) and hamster PRPs for tannin.** Increasing amounts of competitor were mixed with 100 μg of ^{14}C-labeled bovine serum albumin in 640 μl of 0.2 M acetate buffer (pH 4.8). To this was added 160 μl of methanol containing 20 μg of tannin. After centrifugation, supernatant fluids were removed and discarded. The pellets were washed, dissolved in 100 μl of 1% SDS, and assayed for radioactivity by scintillation counting. Inhibition of binding was calculated as described (14). Competitor proteins added were hamster PRPs HP89 (■), HP45 (△), HP43a (▲), HP43b (○), and bovine serum albumin (●).

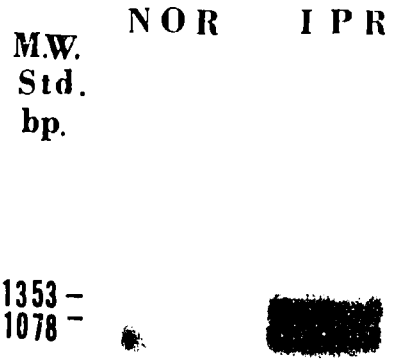

FIG. 7. **Northern hybridization analysis of RNA from parotid glands of normal and isoproterenol-treated hamsters.** RNAs (10 μg) from parotid glands of normal (*NOR*) and isoproterenol treated (*IPR*) were electrophoresed, blotted, and hybridized to ^{32}P-labeled probes. *M.W. Std. bp.*, molecular weight standards (base pairs), prepared by a *Hae*III digest of *X*-174 DNA.

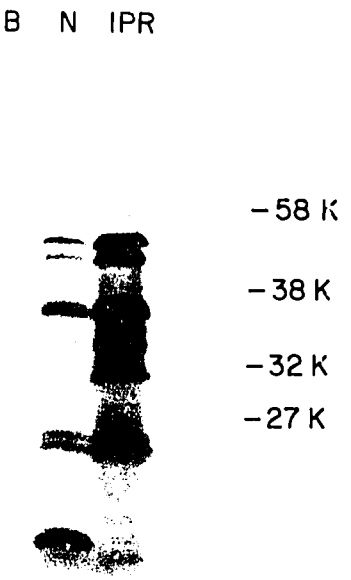

FIG. 6. **Cell-free translations of mRNAs from hamster parotid glands.** Translations were carried out with the reticulocyte lysate system and labeled with [^{3}H]proline. About 5 μl of each translation mixture was analyzed by fluorography. *B*, no added RNA; *N*, normal; *IPR*, isoproterenol treated.

TABLE III

Effects on hamster salivary glands of feeding sorghum with high tannin levels

Diet[a]	Gland weight		Acid-soluble proteins	
	Parotid	Submandibular	Parotid	Submandibular
	g		*mg/g tissue*	
RS-610	0.24	0.46	4.1	11.0
Savanna	0.23	0.45	4.6	12.2

[a] Hamsters were maintained either on RS-610 (low tannin sorghum) or on Savanna (high tannin sorghum) diets for 3 days.

provided by the gelatin and suggests the formation of a tannin-gelatin complex. In contrast, rats maintained on high tannin diets (2% tannin) survived and gained weight after an initial weight loss (13); the ability to gain weight was clearly the result of induction of PRP synthesis by tannins (13). Undoubtedly the severe adverse effects to hamsters of diets high in tannins are due to the lack of induction of the tissue-specific PRP multigene family. Increasing the tannin content of the high tannin diet from 2 to 4% by adding Quebracho

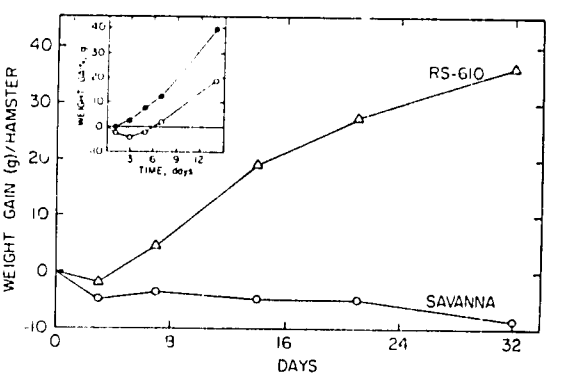

FIG. 8. **Effects of high tannin (Savanna) and low tannin (RS-610) sorghum diets on rate of growth of hamsters.** *Inset*, rate of growth of rats fed Savanna (○——○) and RS-610 (●——●) (13).

TABLE IV

Effects of tannins, gelatin, Quebracho, and an amino acid mix on growth rate of hamsters

Diet	Weight gain[a]		
	7 days	14 days	21 days
		g	
RS-610 (low tannin sorghum)	3.0	14.5	22.7
RS-610 + gelatin (4%)	7.1	20.1	29.3
RS-610 + amino acid mix (4%)[b]	10.4	24.2	28.2
RS-610 + Quebracho (4%)[c]	−20.0 (1)[d]	−24.8 (2)	−28.7
Savanna (high tannin sorghum)	−8.7	−9.0	−9.7
Savanna + gelatin (4%)	4.5	16.0	22.8
Savanna + amino acid mix (4%)	−6.6	−4.6	−3.0
Savanna + Quebracho (4%)	−19.0 (2)	−26.3 (3)	(1)

[a] Weight gains are averages of six hamsters unless indicated.
[b] Amino acid mix was composed as equivalent to the amino acid composition of gelatin.
[c] Quebracho extract.
[d] Numbers in parentheses indicate animals that died.

TABLE V

β-Receptors and adenylate cyclase activities

	^{125}I bound[a]	cAMP synthesized
	cpm/mg protein	*pmol/mg protein*
Membranes		
Rat parotid	10.3×10^5	18.5
Hamster parotid	6.7×10^5	13.5

[a] ^{125}I-Hydroxybenzylpindolol was used as a ligand.

extract was extremely toxic to hamsters (Table IV), and within 3 days half of the animals died.

Assays for β-Receptors and Adenylate Cyclase Activity—The tannin-mediated induction of PRPs in rats and mice was completely blocked by the β-agonist propranolol (25). Since hamsters failed to respond to tannins, it was possible that hamster parotid glands have fewer β-receptors and less adenylate cyclase activity. Results obtained from ^{125}I-hydroxybenzylpindolol binding studies clearly showed that membrane preparations from both rat and hamster parotid glands contained essentially the same number of β-receptors (Table V). Moreover, membrane preparations from rat and hamster parotid glands formed 18.5 and 13.5 pmol of cyclic AMP, respectively, per mg of protein (Table V). Thus, the failure of hamster parotid glands to respond to tannins is not due to either a decrease in adenylate cyclase activity or to fewer β-receptors.

DISCUSSION

Proline-rich proteins and glycoproteins are specifically synthesized and secreted by salivary glands of various animals. In addition to the hamster PRPs reported here, these unusual proteins have been isolated and characterized from human (see Ref. 2 for a review), rat (3, 4), mouse (5, 8), monkey (26), and rabbit (27). Mainly as a result of work from our laboratory it is now known that PRPs of mice, rats, and hamsters are tissue-specific products of multigene families which are dramatically induced by isoproterenol. These unusual proteins pose a number of interesting problems concerning gene structure and evolution, regulation of gene expression, post-translational modifications, and protein conformation.

Ann *et al.* (11) reported the complete sequence of hamster PRP gene H29. The amino acid composition of the derived polypeptide encoded by H29 (Fig. 9) is compared to those of HP43a and HP43b (Table II). The derived sequence of one reading frame from exon II of H29 is Ala-Thr-Ile-Tyr-Glu-Asp-Ser-Ile-Ser-Gln-Leu-Ser (11) which is identical to the N-terminal sequence of HP43a (Fig. 3) except in position 8 (aspartate instead of isoleucine) (Fig. 3). The amino acid sequence of HP43a is blank in position 9, and the derived sequence from H29 has a serine in this position. Therefore, this serine is likely phosphorylated or glycosylated. This same reading frame also encodes a polypeptide of 5 tandemly repeated peptides of amino acid sequence Pro-Pro-Gln-Gln-Glu-Gly-Gln-Gln-Gln-Asn-Arg-Pro-Pro-Lys-Pro-Gly-Asn-Gln-Glu-Gly (Fig. 9 (11)). If HP43a is encoded by H29, it would contain 19 glutamates in 169 amino acids, which would explain why it is an excellent substrate for *S. aureus* V8 (Fig. 4, *lane 2*). From residues 53–63 (Fig. 9, Asp-Glu-Glu-Gly-Asp-Asp-Asp-Gly-Glu-Glu-Asp), nine out of eleven amino acids are acidic, identifying the polypeptide encode by H29 as an acidic PRP. Based on the derived amino acid sequence, there is only one chymotrypsin site among 169 amino acids (F-45) which is consistent with the protease studies of HP43a (Fig. 4, *lane 5*). From the results obtained so far (composition analysis, acidic nature, N-terminal sequence analysis, and sensitivity to proteases), we concluded that H29 codes for HP43a or a very closely related protein.

Northern analysis (Fig. 7) showed two size classes of messages, 1100 and 850 bases, which is common to rat and mouse (6). The calculated size of hamster PRP mRNA encoded by H29 is 727 nucleotides plus the poly(A) tail. Possibly a message of 850 bases is encoded by H29. The wide range in sizes of induced PRPs (25–55 kDa, Fig. 6) cannot be explained by only two size classes of messages, and the anomalous behavior of PRPs on SDS-PAGE as demonstrated for translation products of rat PRP mRNAs (7) likely occurs with the hamster.

The hamster basic PRP HP45 was not phosphorylated (Table II), which is different from human basic PRP IB-1 (24). Also there was no N-terminal homology of HP45 with other basic PRPs from rat, mouse, and human. On the other hand, N-terminal sequences among human, rat, and mouse acidic PRPs, and hamster acidic PRPs HP43a and HP43b are quite conserved (Fig. 3). As we reported previously (11), the evolution of the N-terminal region of acidic PRPs is apparently under negative selection against replacement substitution. In other words, the N-terminal regions of basic PRPs are under less stringent functional constraint than those of acidic PRPs. Recently, Braunlin *et al.* (28) reported that the N-terminal fragment of a human acidic PRP is involved in calcium binding, and this might explain the functional constraint.

With regard to regulation of PRP gene expressions, a different picture is presented by hamsters: (i) hamsters respond to isoproterenol treatment by an increased synthesis of PRPs (Figs. 1, 6, and 7), but there is little, if any, hypertrophic response (Table I) when compared to mice and rats; (ii) feeding tannins has essentially no effect on hamster salivary glands (Table III); (iii) hamsters fed a diet containing 2% tannin lose weight for about 3 days, as do rats (13) and mice (5), but then an unusual continued growth inhibition is ob-

FIG. 9. **Amino acid sequence derived from PRP gene H29.** Repeat region arranged to align homologous sequences.

served (Fig. 8). Hamsters maintained on 2% tannin diets failed to grow and even at 60 days were essentially at the same body weight as at 3 days. When the diets are switched, the experimental animals gained weight at almost the normal rate for young hamsters, while the control animals, now on a 2% tannin diet, lost about 20% of their weight. In about 20 days, both groups of hamsters were close to the same weight. Hamsters' PRPs have a similar affinity to tannin when compared to those of rat and mouse (Fig. 5) so this unusual growth inhibition cannot be explained by the failure of hamster PRPs to bind tannins. Clearly, the detrimental effects of tannins (see Ref. 29 for a review) were reversed by the induction of PRPs in rats (13) and mice (5). Hamsters are unusually susceptible to tannins, but this can be reversed by addition of gelatin to the diet (Table IV). Increasing the tannin content of the diet to 4% was fatal to hamsters with many animals dying within 3 days. There were no differences in the number of β-receptors and the activity of adenyl cyclase (Table V) between hamster and rat parotid glands, and there were several strong conservations at the nucleotide level of 5′-flanking regions between hamster and mouse PRP genes (11), including cAMP-inducible elements.

Tannins ingested in large amounts, used as treatment for burns and as adjuvant for barium enemas, can cause carcinomas, hepatotoxicity, and apparently other pathological and toxic problems (see Ref. 29 for a review). We believe that the PRPs in saliva constitute the first-line defense against tannins ingested. Dietary tannins and their induction and interactions with PRPs have recently been reviewed (29). A mechanism for regulation of the expression of the PRP multigene families by isoproterenol and by tannins undoubtedly is to modulate levels of cAMP through activation of β-receptors in the parotid glands. The mode of inducing a β-agonist by dietary tannins is as yet unknown. Experiments using cell transfections with mouse and hamster PRP genes with different 5′-upstream deletions have identified the nucleotide sequence inducible by cAMP, isoproterenol, or forskolin as ATGTAACAGTCA.[*] The underlined G is missing in hamster PRP gene H29 (11). This sequence is missing from the 5′-upstream region of the human PRP genes (12) and likely explains the induction of PRP genes in mice, rats, and hamsters and the constitutive nature in humans.

The response in the case of mice and rats or the lack of response in the case of hamsters to dietary tannins is likely modulated by events in the gastrointestinal tract. Addition of the β-blocker propranolol to the diet blocks the effects of tannins on the parotid glands of rats and mice (29). Data on β-receptors and adenylcyclase suggest that induction of PRPs in hamsters is modulated by β-agonists and β-receptors. Information on transfections with PRP genes MP2 (6) and H29 (11) and with gene deletions of the 5′-upstream region of these genes clearly shows that cAMP is at least one causative agent. Both MP2 and H29 have almost identical upstream regulatory sequences so that the lack of regulation of expression of the hamster PRP multigene family by tannins apparently is not at the glandular level. The unusual growth-inhibiting effects of hamsters by tannins do not result in stunting, at least not within 6 months of feeding, since these animals responded to a change in diet by exhibiting essentially normal growth. Effects on hormones regulating growth patterns need to be investigated.

[*] P. S. Wright, D. K. Ann, and D. M. Carlson, unpublished observations.

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REFERENCES

1. Carlson, D. M., Ann, D. K., and Mehansho, H. (1986) *Microbiology (Wash. D. C.)* 303–306
2. Bennick, A. (1982) *Mol. Cell. Biochem.* **45,** 83–99
3. Muenzer, J., Bildstein, C., Gleason, M., and Carlson, D. M. (1979) *J. Biol. Chem.* **254,** 5623–5628
4. Mehansho, H., and Carlson, D. M. (1983) *J. Biol. Chem.* **258,** 6616–6620
5. Mehansho, H., Clements, S., Sheares, B. T., Smith, S., and Carlson, D. M. (1985) *J. Biol. Chem.* **260,** 4418–4423
6. Ann, D. K., Clements, S., Johnstone, E., and Carlson, D. M. (1987) *J. Biol. Chem.* **262,** 899–904
7. Ziemer, M. A., Swain, W. F., Rutter, W. J., Clements, S., Ann, D. K., and Carlson, D. M. (1984) *J. Biol. Chem.* **259,** 10475–10480
8. Clements, S., Mehansho, H., and Carlson, D. M. (1985) *J. Biol. Chem.* **260,** 13471–13477
9. Maeda, N., Kim, H-S., Azen, E. A., and Smithies, O. (1985) *J. Biol. Chem.* **260,** 11123–11130
10. Ann, D. K., and Carlson, D. M. (1985) *J. Biol. Chem.* **260,** 15863–15872
11. Ann, D. K., Gadbois, D., and Carlson, D. M. (1987) *J. Biol. Chem.* **262,** 3958–3963
12. Kim, H-S., and Maeda, N. (1986) *J. Biol. Chem.* **261,** 6712–6718
13. Mehansho, H., Hagerman, A., Clements, S., Butler, L., Rogler, J., and Carlson, D. M. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 3948–3952
14. Hagerman, A. E., and Butler, L. G. (1981) *J. Biol. Chem.* **256,** 4494–4497
15. Ludford, J. M., and Talamo, B. R. (1980) *J. Biol. Chem.* **255,** 4619–4627
16. Ludford, J. M., and Talamo, B. R. (1983) *J. Biol. Chem.* **258,** 4831–4838
17. Tovey, K. C., Oldham, K. G., and Whelan, J. A. M.. (1974) *Clin. Chim. Acta* **56,** 221–234
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
19. Hermodson, M., Schmer, G., and Kurachi, K. (1977) *J. Biol. Chem.* **252,** 6276–6279
20. Gross, E., and Witkop, B. (1962) *J. Biol. Chem.* **237,** 1856–1860
21. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299
22. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 5201–5205
23. Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. (1977) *Biochemistry* **16,** 4743–4751
24. Kauffman, D., Hofmann, T., Bennick, A., and Keller, P. (1986) *Biochemistry* **25,** 2387–2392
25. Asquith, T., Mehansho, H., Rogler, J., Butler, L., and Carlson, D. M. (1985) *Fed. Proc.* **44,** 4016
26. Oppenheim, F. G., Offner, G. D., and Troxler, R. F. (1985) *J. Biol. Chem.* **260,** 10671–10679
27. Rajan, A. I., and Bennick, A. (1983) *Arch. Oral Biol.* **28,** 431–439
28. Braunlin, W. H., Vogel, H. J., Drakenberg, T., and Bennick, A. (1986) *Biochemistry* **25,** 584–589
29. Mehansho, H., Butler, L. G., and Carlson, D. M. (1987) *Annu. Rev. Nutr.*, in press