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ELIMINATION OF ISOPROTERENOL-INDUCED PROLINE-RICH PROTEIN BIOSYNTHESIS IN RAT SALIVARY GLANDS AFTER ADULT THYROIDECTOMY

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Abstract—Surgical thyroidectomy of adult rats resulted in a gradual decrease in β-adrenergic receptor density on the cell surface of parotid and submandibular glands. The decrease in β-adrenergic receptors was 29 and 50% by 2 and 4 weeks, respectively, for the parotid gland. In the submandibular gland, a decrease of 50% of the total β-receptor density was evident after 2 weeks. After 4 weeks no further decrease in β-adrenergic receptor was observed. Subsequent challenge of the salivary glands with chronic treatment of isoproterenol (β-adrenergic receptor agonist) failed to induce proline-rich protein and glycoprotein biosynthesis in the submandibular gland 2 weeks after thyroidectomy, whereas the parotid gland showed induced proline-rich protein but not the glycoprotein synthesis. By 4 weeks the parotid gland did not show the induced synthesis of the glycoprotein or proline-rich proteins. The inability to induce protein synthesis was reflected by decreased cAMP accumulation in both glands after injection with isoproterenol. Partial reversal of these effects on protein synthesis and cAMP accumulation was obtained by triiodothyronine treatment of thyroidectomized rats.

Evidence presented by several laboratories suggests that the level of the thyroid hormone triiodothyronine (T_3†) affects physiological responses of a variety of tissues in a manner similar to those induced by β-adrenergic stimulation [1–6]. Williams *et al.* [7] have shown that increased thyroid hormone levels due to hyperthyroidism result in increased numbers of β-adrenergic receptors on rat myocardium. The administration of either T_3 or thyroxine to rats results in similar observations of increased β-receptor density and increased sensitivity when exposed to β-adrenergic receptor agonists. The increased responsiveness of rat cardiac tissue to β-agonist challenge, for instance, could be blocked by the additional administration of β-receptor antagonists.

The administration of thyroxine to neonatal rats leads to the precocious morphological development of the parotid and submandibular glands in a manner similar to that reported to occur with chronic isoproterenol administration [6, 8–11]. In addition, under conditions of chronic administration of thyroxine to neonatal rats, the induced synthesis of two enzymes, prolidase and prolinase, was observed in the parotid and submandibular gland [6]. Medina *et al.* [15] have shown that surgical removal of the thyroid gland leads to decreased levels of β-receptors on the submandibular gland and subsequent decreases in isoproterenol-evoked salivary secretion in the adult rat. Previous reports from our laboratory have indicated that changes in the β-agonist stimulated levels of cAMP affect the induction of synthesis of salivary gland proline-rich proteins and glycoproteins [12]. Changes in cAMP concentration and gene expression in the rat salivary gland are mediated by interaction with surface β-receptors.

The proline-rich proteins and glycoproteins are not normally produced in significant quantities in rat salivary glands. However, chronic administration of isoproterenol causes *de novo* synthesis of these proteins in both the parotid and submandibular glands [13–16]. After a 10-day drug regimen, these proteins have been shown to constitute 50% of the protein in rat saliva.

In the present paper we examined the effect of thyroidectomy on β-adrenergic receptor density and ability of chronic isoproterenol challenges to induce proline-rich protein and glycoprotein synthesis in rat salivary glands. Decreased receptor density on the cell surfaces of both the parotid and submandibular gland resulted in lower levels of cAMP accumulation in these glands after challenge with isoproterenol, with the concomitant loss of induced protein biosynthesis. Partial reversal of the effects of surgical thyroidectomy on the response of the salivary gland to isoproterenol treatment was obtained by the administration of T_3. The exogenously administered hormone caused increased cell β-adrenergic receptor density and higher levels of cAMP accumulation when challenged with isoproterenol.

MATERIALS AND METHODS

Chemicals. *d,l*-Isoproterenol, T_3 and alprenolol were purchased from the Sigma Chemical Co. [^{3}H]Dihydroalprenolol (sp. act. 30–60 Ci/mmol) and [^{3}H]cAMP (sp. act. 300 Ci/mmol) and [^{125}I]tri-

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† Abbreviations: T_3, triiodothyronine; SDS, sodium dodecyl sulfate; cAMP, cyclic AMP; DHP, dihydroalprenolol; TCA, trichloroacetic acid; and ISO, isoproterenol.

iodothyronine assay kits were purchased from Amersham. Ultrapure reagents for polyacrylamide gel electrophoresis were purchased from Bio-Rad. All other chemicals were purchased through commercial sources and were of reagent grade quality.

Preparation of animals. Male Wistar rats (200 g) for these experiments were purchased from Charles River Animal Farms. Surgical thyroidectomy along with sham thyroidectomy controls were performed before purchase at 7 weeks of age. The animals were given laboratory chow and water, supplemented with 10 mM $CaCl_2$, *ad lib.* for 2 to 4 weeks prior to the initiation of isoproterenol treatment. Isoproterenol was administered intraperitoneally at 6 mg/200 g animal twice daily for 10 days. A third group of animals was given subcutaneous injections of 10 mg/100 g body weight T_3 for 14 days.

Tissue preparation and protein isolation. Parotid and submandibular glands were identified by gross morphology. The glands were removed from animals that had been anesthetized with sodium pentobarbital and killed by exsanguination. The soluble and insoluble membrane fractions were prepared by homogenization in 10 vol. of 50 mM Tris–HCl, pH 7.4, 10 mM $MgCl_2$ and 0.32 M sucrose, with a Dounce apparatus. The slurry was centrifuged at 1,000 *g* for 5 min. The supernatant fraction was recovered and centrifuged at 30,000 *g* for 30 min. The resulting pellet was resuspended in cold buffer for later use in receptor assays, while the supernatant fraction was precipitated by an equal volume of 12% TCA; removal of the TCA was accomplished as described previously by Mehancho and Carlson [16]. The cleared supernatant fraction was subsequently used in cAMP assays and for the purification of the TCA soluble proline-rich proteins and glycoproteins as described elsewhere [8, 12]. Protein assays on all samples were performed by a modification [17] of the Lowry method, with bovine serum (Sigma Chemical Co.) as a standard.

Serum levels of T_3 were determined by radioimmunoassay using a kit assay system from Amersham containing [^{125}I]triiodothyronine (sp. act. > 1200 5 Ci/μg). The serum samples were obtained by cardiac puncture 12 hr after the final injection of the drug.

Polyacrylamide gel electrophoresis and protein staining. Protein samples were subjected to electrophoresis in 0.75-mm thick 10% (w/v) polyacrylamide gels by using a modified Tris/glycine Laemmli system described by Pugsley and Schnaitman [18]. All gels were fixed and stained by a modification of the method by Fairbanks *et al.* [19] as described elsewhere [20]. Samples for gels were made up to 1 mg protein/ml sample buffer. A 35-μg sample of protein per well was routinely used in subsequent electrophoresis unless stated otherwise. Proline-rich glycoproteins were stained for carbohydrate by using the periodate/Schiff reagent procedure of Fairbanks *et al.* [19] without modification. Radioligand binding of [^{3}H]dihydroalprenolol (DHP) β-adrenergic receptors were assayed by using [^{3}H]DHP as radioligand as described by Ludford and Talamo [21] and Medina *et al.* [5]. Each sample was run in duplicate using 300–500 μg of membrane protein [5], in a 1-ml volume containing 0.1 to 2.0 nM [^{3}H]DHP. The reactions were carried out at 37° for 30 min, and the reaction was terminated by the addition of 3 ml of 0.9% NaCl and placement at 4°. Sample results were obtained by collecting the cell membranes on Whatman GF/B filters, washing three times with additional saline solution, and determining radioligand binding by scintillation counting using a non-aqueous mixture and a tritium window for measuring [^{3}H]DHP cpm. Non-specific binding was determined by preincubation of membranes with 10 μM propranolol before the addition of DHP to the reaction mixture. Scatchard plots were evaluated by linear regression to calculate the K_D of [^{3}H]DHP binding and the number of binding sites.

Measurement of adenosine 3′:5′-monophosphate levels. Cyclic AMP levels were determined by the method of Gilman [22] using an Amersham assay kit. The assays were performed on fresh gland preparations prepared 10 min after isoproterenol treatment as described by Grand and Schay [23] and Wells and Humphreys-Beher [12].

Radioimmunoassay of T_3 levels. The serum levels of T_3 were determined using the Amersham radioimmunoassay kit. Four milliliters of blood obtained by cardiac puncture was allowed to coagulate, and the serum was collected after centrifugation at 500 *g* for 5 min. This assay depends upon the competition for binding of an antibody against thyronine with cold serum T_3 and a known concentration of ^{125}I-labeled T_3 added to each reaction. Duplicate reactions of 25 μl of sample serum were assayed in determining hormone levels.

RESULTS

Quantitative changes in β-adrenergic receptor density. The induction of protein synthesis in rat salivary glands by isoproterenol is dependent on the interaction with β-adrenergic receptors of the cell surface [14, 15]. *De novo* protein biosynthesis has been shown to take place subsequent to transient increases in cellular cAMP concentrations after β-agonist stimulation [13]. To explore this relationship further, rats were subjected to surgical removal of their thyroid gland and assayed for changes in β-receptor density. β-Adrenergic receptor densities were determined by the binding of the β-adrenergic antagonist [^{3}H]dihydroalprenolol. As shown in Table 1, thyroidectomy of adult animals resulted in a 29 and 50% loss of β-receptors from parotid and submandibular membranes, respectively, after 2 weeks. After the thyroid gland had been removed for 4 weeks, the decrease in receptor density was approximately 50% of sham-operated control animals for both glands, and it did not change further with time. During this same period, there was a corresponding decrease in circulating serum levels of the thyroid hormone, T_3, of approximately 67% when compared to sham-thyroidectomized controls. The administration of triiodothyronine increased serum levels of the hormone in animals that had undergone prior surgical thyroidectomy. The increased T_3 in these animals resulted in partial restoration of the β-adrenergic receptors on the parotid and submandibular gland cell surfaces (Table 1).

The decrease in [^{3}H]DHP binding was further

Table 1. Effects of thyroidectomy on β-adrenergic receptor density and cAMP in response to isoproterenol treatment of salivary glands

Gland	Treatment	β-Adrenergic receptor densities*	cAMP levels†	Serum T_3 levels‡
Parotid				
	Sham§	119.7 ± 5.9	5.9 ± 1.4	89.6 ± 5.2
	Sham/ISO	114.6 ± 3.8	87.4 ± 3.0	85.7 ± 8.1
	2-week Thyroidectomy/ISO	85.9 ± 6.1	80.6 ± 2.9	47.1 ± 6.3
	4-week Thyroidectomy/ISO	62.4 ± 4.0	43.7 ± 4.2	30.2 ± 4.7
	6-week Thyroidectomy	64.5 ± 3.9	5.5 ± 2.1	32.7 ± 4.0
	4-week Thyroidectomy T_3	92.0 ± 4.7	6.0 ± 3.2	50.1 ± 5.1
	4-week Thyroidectomy T_3/ISO	97.4 ± 5.1	73.4 ± 3.7	51.7 ± 4.9
Submandibular				
	Sham	75.9 ± 5.9	3.8 ± 1.5	89.6 ± 5.2
	Sham/ISO	74.4 ± 5.2	52.9 ± 1.9	85.7 ± 8.1
	2-week Thyroidectomy/ISO	36.3 ± 3.6	17.3 ± 2.3	47.1 ± 6.3
	4-week Thyroidectomy/ISO	31.4 ± 4.1	15.7 ± 5.1	30.2 ± 4.7
	6-week Thyroidectomy	31.0 ± 3.9	3.2 ± 2.5	32.7 ± 4.0
	4-week Thyroidectomy T_3	43.6 ± 5.5	3.5 ± 1.9	50.1 ± 5.1
	4-week Thyroidectomy T_3/ISO	47.2 ± 3.5	36.8 ± 2.2	54.6 ± 6.1

* β-Adrenergic receptor density was determined by [^{3}H]dihydroalprenolol binding to membrane preparations of the parotid and submandibular gland. All values are B_{max}, expressed as fmoles [^{3}H]DHP bound/mg membrane protein ±1 SD and are the result of duplicate assays performed with six experimental animals.

† cAMP was measured using an assay kit purchased from Amersham. Results of duplicate assays on six experimental animals are expressed as pmoles cAMP/mg total cell protein ±1 SD. cAMP was assayed 15 min subsequent to ISO challenge as described previously [12].

‡ Values are expressed as ng/100 ml for T_3. The results (mean ± 1SD) are based on duplicate determinations of assays performed on six animals.

§ Sham thyroidectomy refers to control animals that have undergone the same surgical procedures as the experimental group; however, the thyroid gland was not removed.

characterized for changes in receptor affinity rather than decreased surface density. As shown in Fig. 1, when the binding of radiolabeled antagonist was examined by Scatchard analysis of saturation curves, the dissociation constant, K_D, was 0.85 ± 0.1, 0.81 ± 0.13 and 0.78 ± 0.1 nm for parotid gland cells from sham-thyroidectomy control, 2-week thyroidectomy and 4-week thyroidectomy respectively. The same dissociation constant value for K_D was obtained using cell membranes isolated from the submandibular gland (data not shown). From these results, it is evident that alterations in the thyroid hormone balance modify the density of β-adrenergic receptors without apparent changes in the affinity of these receptors from the parotid and submandibular glands.

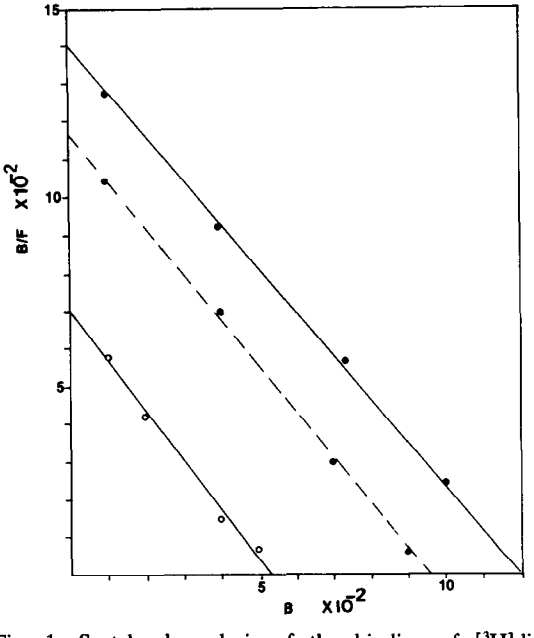

Fig. 1. Scatchard analysis of the binding of [^{3}H]dihydroalprenolol to parotid gland cell membrane fractions. Key: Solid line with closed circles, sham thyroidectomy; dash line, 2-week thyroidectomy; solid line with open circles, 4-week thyroidectomy. B = bound ligand expressed as fmol/mg protein; F = free ligand expressed as nM.

Induction of protein synthesis. The change in β-adrenergic receptor density was examined for an effect on protein synthesis induced by chronic isoproterenol treatment. Chronic isoproterenol treatment of rats results in the *de novo* biosynthesis of previously characterized proline-rich proteins and glycoproteins in the parotid and submandibular glands [13–16]. Two and four weeks after surgical thyroidectomy, rats were given a 10-day regimen of daily injections of isoproterenol, and the synthesis of proline-rich proteins was examined by polyacrylamide gel electrophoresis. As shown in Fig. 2, the ability of isoproterenol to affect *de novo* biosynthesis of these proteins appeared to be dependent on the density of surface β-adrenergic receptors on cells of the parotid and submandibular glands (lanes 4, 5, 8 and 9). Proteins from control and isoproterenol-treated sham-thyroidectomized rat parotid glands are represented in lanes 2 and 3 respectively. As previously reported, the proteins of 200,000

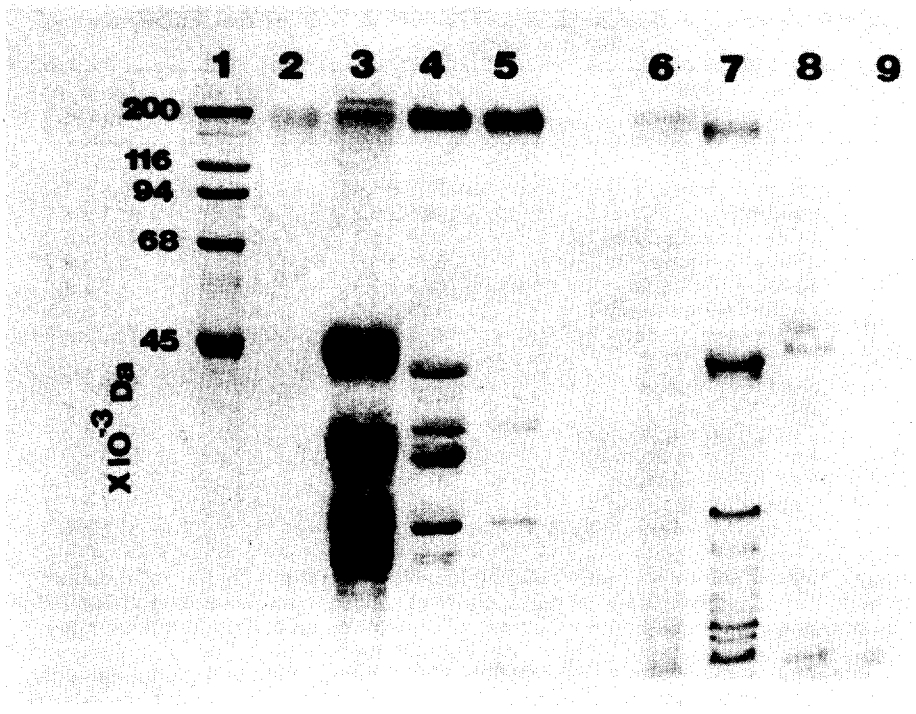

Fig. 2. Ten percent SDS–polyacrylamide gel of parotid and submandibular gland TCA soluble protein extracts from sham and thyroidectomized animals treated with isoproterenol for 10 days. Lane 1 (molecular weight standards): 200,000 daltons, myosin; 116,000 daltons, β-galactosidase; 94,000 daltons, phosphorylase B; 68,000 daltons, bovine serum albumin; and 45,000 daltons, ovalbumin; lane 2: sham thyroidectomy parotid gland; lane 3: sham thyroidectomy isoproterenol-treated parotid gland; lane 4: 2-week thyroidectomy isoproterenol-treated parotid gland; lane 5: 4-week thyroidectomy isoproterenol-treated parotid gland; lane 6: sham thyroidectomy submandibular gland; lane 7: sham thyroidectomy isoproterenol-treated submandibular gland; lane 8: 2-week thyroidectomy isoproterenol-treated submandibular gland; and lane 9: 4-week thyroidectomy isoproterenol-treated submandibular gland. Proline-rich proteins were recovered from the TCA soluble fraction by dialysis against 5% acetic acid followed by lyophilization. The proline-rich proteins are those proteins previously identified as migrating in polyacrylamide gels with molecular weights between 45,000 and 17,000 daltons. Approximately 35 μg of protein was loaded into each sample well and stained for total protein by Coomassie Brilliant blue R-250.

and 220,000 daltons were identified as the parotid gland proline-rich glycoproteins [20]. Lane 6 and 7 are the corresponding proteins from the submandibular glands of sham-thyroidectomized animals. The glycoproteins previously identified by PAS staining that are synthesized by this gland have apparent molecular weights of 200,000, 190,000 and 180,000 daltons ([20]; data not presented). Nonglycosylated proline-rich proteins have been identified as having electrophoretic mobilities of 45,000 to 17,000 daltons in both the parotid and submandibular glands. When a constant amount of protein was examined after SDS–polyacrylamide gel electrophoresis from isoproterenol-treated parotid glands of thyroidectomized animals, there was an evident decrease in the inducible synthesis of the proline-rich proteins (lanes 4 and 5). In addition to giving the appearance of increased synthesis of the 200,000 dalton glycoprotein, the changes in the relative concentrations of the lower molecular weight proteins suggest possible changes in protein mobility. These differences are, however, an artifact of running constant protein levels in these gels.

An examination of the submandibular gland showed that adult thyroidectomy resulted in the cessation of synthesis of the 200,000 and 180,000 dalton glycoproteins that are produced in the untreated animal. It is also evident from these results that, although two proteins appear to be induced in the isoproterenol-treated submandibular glands of thyroidectomized animals (45,000 and 50,000 daltons), they do not correspond to the proline-rich proteins present in the sham isoproterenol-treated animal.

The results of isoproterenol-induced protein synthesis in the parotid and submandibular glands show differences in sensitivity to decreased β-receptor density as a consequence of surgical thyroidectomy. The submandibular gland could be shown to lack induced protein synthesis with the 50% loss of surface β-receptors after 2 weeks of thyroidectomy, whereas the parotid gland retained the ability to synthesize proline-rich proteins (apparent molecular weight 45,000 to 17,000 daltons) with a 30% loss of surface β-adrenergic receptors (Fig. 2 and Table 1). However, the parotid gland at 2 weeks post-thyroidectomy had lost the ability to produce the 220,000 dalton glycoprotein normally induced by chronic isoproterenol treatment. By 4 weeks after thyroidectomy, the parotid gland had also lost the ability to respond to isoproterenol treatment as

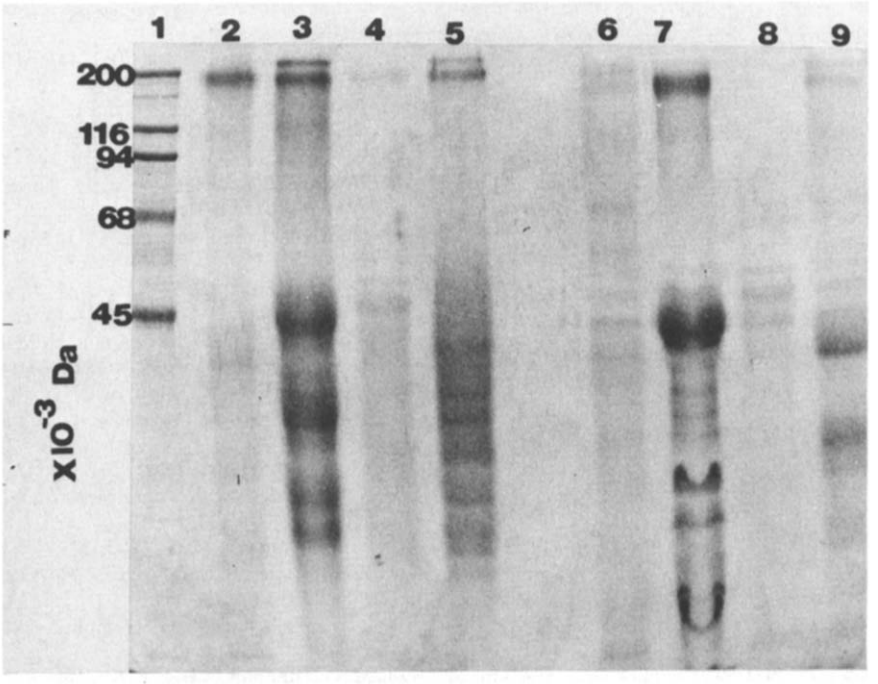

Fig. 3. Ten percent SDS–polyacrylamide gel of 10% TCA soluble protein from control and 4-week thyroidectomized salivary glands of animals supplemented with exogenous thyroid hormone, T_3, for 14 days. Animals were subsequently introduced to a 10-day regimen of daily isoproterenol injections. Lane 1: molecular weight standards, weights as in Fig. 2; lane 2: untreated control sham thyroidectomy parotid gland; lane 3: isoproterenol-treated control parotid gland; lane 4: 4-week thyroidectomized animal provided with 14-day T_3 supplements, parotid gland; lane 5: 4-week thyroidectomized animal, isoproterenol-treated parotid gland supplemented with T_3; lane 6: untreated control submandibular gland; lane 7, isoproterenol-treated submandibular gland; lane 8, 4-week thyroidectomized animal submandibular gland provided with 14-day T_3 supplements; and lane 9: 4-week thyroidectomized animal submandibular gland supplemented with T_3 for 14 days prior to chronic isoproterenol treatment. All samples represent proline-rich proteins isolated from the TCA soluble material of gland extracts; 35 μg of protein was loaded per sample well. Gels were stained for total protein by Coomassie Brilliant Blue R-250.

judged by the lack of inducible proline-rich protein synthesis, although there may have been several of the proline-rich proteins still present (see Fig. 2). During this time, the parotid gland continued to show a decrease in the level of surface β-adrenergic receptor density. In both the parotid and submandibular glands, the decline in β-adrenergic receptors was accompanied by a corresponding decrease in cellular cAMP concentrations found immediately after challenge with isoproterenol. It should be noted, however, that basal levels of cAMP remained constant in all animals (Table 1). Previous results from our laboratory have suggested that the synthesis of proline-rich proteins and glycoproteins of the salivary glands is under a type of threshold response to the accumulation of cellular cAMP [12]. The ability to induce the proline-rich proteins of the parotid gland but not the 220,000 dalton proline-rich glycoprotein upon decreased surface β-adrenergic receptor density and suboptimal cAMP accumulation in the 2-week thyroidectomized animals supports this conclusion.

Introduction of exogenous hormone to rats The exogenous administration of triiodothyronine was provided to thyroidectomized animals in an attempt to reverse the effects on β-adrenergic receptor density and thus restore inducible protein synthesis in the salivary glands. As shown in Fig. 3, upon injection of the thyroid hormone into 4-week thyroidectomized animals for 2 weeks, subsequent challenge with isoproterenol led to a restoration of the ability for parotid and submandibular glands to synthesize these specific proteins. Lanes 2, 3, 6 and 7 are control and isoproterenol-treated parotid and submandibular glands respectively. When isoproterenol was administered to the hormone-treated animals, the parotid gland showed the restoration of the inducible 220,000 dalton glycoprotein (lane 5). There was also an increased ability of isoproterenol to induce the low molecular weight proline-rich proteins which are not present in the parotid glands of animals receiving triiodothyronine alone (lane 4). It is interesting to note that, whereas the thyroidectomized submandibular gland treated with triiodothyronine did not produce the 200,000 and 180,000 dalton glycoproteins, challenge with isoproterenol restored their synthesis and that of the non-glycosylated proline-rich proteins but not the isoproterenol-induced 190,000 dalton glycoprotein (lanes 8 and 9). The ability of isoproterenol to again influence salivary gland protein synthesis was reflected by the exogenously administered hormone to increase β-receptors

on the cell surface from the salivary glands (Table 1). This resulted in the concomitant increased accumulation of cAMP relative to that level observed in the experimental animals with no treatment (Table 1).

DISCUSSION

The results presented in this paper provide evidence that β-adrenergic receptors on the necessary parotid and submandibular glands are necessary in order for isoproterenol to influence gene expression. As has been shown previously for other tissues [1–5], β-receptor density in the salivary glands of rat is influenced by the levels of circulating thyroid hormone. Removal of the thyroid gland resulted in a diminished number of β-adrenergic receptors, which influence isoproterenol-mediated salivary gland gene expression. This finding is in agreement with the results of Medina *et al.* [5] where thyroidectomy produced a decrease in isoproterenol-stimulated salivary secretion from the submandibular gland. However, no further characterization of protein synthesis was performed involving long-term exposure to isoproterenol.

The interaction of β-adrenergic agonists with β-receptors causes transient increases in cellular cAMP levels [12, 23]. The apparent intracellular concentration of cAMP after β-agonist treatment plays a role in gene expression in the parotid gland [12]. The recent sequencing of the mouse genomic DNA for the proline-rich protein gene family has identified putative sequences that have homology to proposed cAMP regulatory regions [24, 25]. Cyclic AMP or cellular metabolite influenced by cAMP concentrations could act as a regulator of gene expression for proline-rich protein biosynthesis. Decreased levels of surface β-adrenergic receptors may, therefore, be expected to affect parotid and submandibular gland proline-rich protein and glycoprotein biosynthesis by lowering the level of cAMP accumulated during β-agonist challenge.

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