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KClO₄ inhibits thyroidal activity in the larval lamprey endostyle in vitro

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Abstract

An in vitro experimental system was devised to assess the direct effects of the goitrogen, potassium perchlorate (KClO₄), on radioiodide uptake and organification by the larval lamprey endostyle. Organification refers to the incorporation of iodide into lamprey thyroglobulin (Tg). Histological and biochemical evidence indicated that endostyles were viable at the termination of a 4 h in vitro incubation. A single iodoprotein, designated as lamprey Tg, was identified in the endostylar homogenates by polyacrylamide gel electrophoresis and Western blotting. Lamprey Tg was immunoreactive with rabbit anti-human Tg serum and had an electrophoretic mobility similar to that of reduced porcine Tg. When KClO₄ was added to the incubation medium, both iodide uptake and organification by the endostyle were significantly reduced relative to controls as determined by gamma counting, and gel-autoradiography and densitometry, respectively. Western blotting showed that KClO₄ significantly lowered the total amount of lamprey Tg in the endostyle. Based on the results of this in vitro investigation, we conclude that KClO₄ acts directly on the larval lamprey endostyle to inhibit thyroidal activity. These data support a previous supposition from in vivo experimentation that KClO₄ acts directly on the endostyle to suppress the synthesis of thyroxine and triiodothyronine, resulting in a decrease in the serum levels of these two hormones.

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1. Introduction

The larval lamprey endostyle, which gives rise to follicular thyroid tissue during metamorphosis, is the site of thyroid hormone (TH) synthesis (Hardisty and Baker, 1982). Experimental, histological, and biochemical evidence support the idea that the mechanism of TH synthesis in the lamprey endostyle is similar to that observed in follicular thyroid tissue. Histological autoradiography at the light and electron microscope levels has shown that radioiodide (¹²⁵I[−]) is concentrated and bound in several endostyle cell types, with cell types 2c and 3 being the primary iodide binding cells (Fig. 1; for review see Barrington and Sage, 1972; Wright and Youson, 1976). These type 2c and type 3 cells are immunoreactive with an antibody against human thyroglobulin (Tg) (Wright et al., 1978) and they have

peroxidase activity (Tsuneki et al., 1983). Biochemical studies with radioiodide indicate that iodide taken up by the endostyle is incorporated into iodoproteins of different sizes and sedimentation coefficients. Included among these iodoproteins are 19S and 12S proteins that correspond to the homodimer and monomer, respectively, of mammalian Tg (Monaco et al., 1978; Suzuki and Kondo, 1973). Furthermore, hydrolysis of these iodoproteins has confirmed that they contain iodothyronines, TH, and the TH precursors monoiodotyrosine (MIT) and diiodotyrosine (DIT) (Salvatore, 1969; Suzuki and Kondo, 1973). These data indicate that the necessary components for TH synthesis, namely, an iodide-concentrating mechanism, a Tg-like molecule, and peroxidase activity, can be localized to particular cell types in the larval lamprey endostyle.

The processes involved in the regulation of TH synthesis by the larval lamprey endostyle are less clear. Studies conducted to date have not confirmed whether TH synthesis and secretion by the larval lamprey endostyle are regulated by the hypothalamic–pituitary axis (Barrington and Sage, 1963a,b, 1966; Clements-Merlini, 1962a; Knowles, 1941; Pickering, 1972). On the other

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Fig. 1. Routine light microscopy (A and C) and autoradiography (B and D) of transverse sections through the anterior portion of the larval lamprey (*Lampetra appendix*) endostyle following a 4 h in vitro incubation with 3 $\mu\text{Ci Na}^{125}\text{I}$. Endostyle cell types 1, 2a, 2b, 2c, 3, and 5 are indicated. (A) The anterior endostyle consists of two straight, epithelium-lined chambers; the most prominent features are the four glandular tracts (Gt), which consist exclusively of type 1 cells. Flanking the openings of the glandular tracts to the lumen of the endostyle are the type 2 cells. Continuous with the type 2c cells are the type 3 endostylar cells. (C) A high magnification of a glandular tract and the surrounding epithelia in the anterior portion of the larval lamprey endostyle. Note that the cells of the epithelia are intact with well-defined cytoplasm and nuclei, confirming that the 4 h incubation with Na^{125}I did not have any visible effects on cellular morphology. (B and D) Light microscopic autoradiography of sections similar to A and C, respectively. The type 2c and 3 cells are the primary $^{125}\text{I}^-$ incorporating cell types. In all four micrographs, the endostyles are oriented with the ventral side on the left. Magnification is $\times 165$ (A and B) and $\times 330$ (C and D). Blood vessels, Bv; Gills, Gi; Pigment, Pi.

hand, anti-thyroid agents (goitrogens), known to inhibit either iodide uptake or organification in follicular thyroid tissue, alter both functional and morphological aspects of the larval endostyle. Overall, however, the data from these goitrogen studies are not consistent (Salvatore, 1969) and are difficult to interpret. Clements-

Merlini (1962b) found that the goitrogens, thiourea and thiocyanate, inhibited radioiodide accumulation and that thiourea inhibited the formation of iodinated tyrosines. Subsequent reports from several studies indicated that thiourea and thiouracil alter the functional morphology of all endostyle cell types including those

not involved in thyroidal activity (Barrington and Sage, 1963a,b, 1966).

More recent studies have focused on the ability of goitrogens to lower serum TH concentrations and induce precocious metamorphosis in lampreys (Hoheisel and Sterba, 1963; Holmes and Youson, 1993; Holmes et al., 1999; Leatherland et al., 1990; Manzon et al., 2001; Suzuki, 1986, 1987; Youson et al., 1995). These studies support the view that spontaneous metamorphosis is linked to a decline in serum TH concentrations (Lintlop and Youson, 1983; Wright and Youson, 1977). Furthermore, Manzon et al. (1998) have conclusively shown that elevating serum TH concentrations with exogenous TH prevents potassium perchlorate (KClO_4)-induced metamorphosis. This result correlates well with the observation that exogenous TH can retard spontaneous metamorphosis (Youson et al., 1997). However, whether goitrogens, such as KClO_4 , bring about a decrease in serum TH concentrations and metamorphosis by either: (i) affecting some process underlying both TH titers and metamorphosis; (ii) a direct effect on the endostyle; or (iii) causing a generalized chronic debilitation, has yet to be determined. A partial answer to these questions could come from a study that determines whether goitrogens can act directly on the endostyle and reduce its capacity to synthesize TH.

Numerous studies have investigated the effects of goitrogens on endostyle morphology, serum TH concentrations, and metamorphosis in larval lampreys. However, little is known about the direct effects that goitrogens have on TH synthesis by the larval endostyle. One aim of this study was to develop an *in vitro* experimental system to investigate the mechanisms through which goitrogens might alter TH synthesis by the larval endostyle and result in the characteristic depression of serum TH concentrations that we have shown following *in vivo* application. Our primary goal was to use this *in vitro* system to determine the effects of KClO_4 on the uptake and organification of iodide by the larval endostyle; however, these data are also valuable to our understanding of KClO_4 -induced metamorphosis in lampreys (see Manzon and Youson, 1997; Manzon et al., 1998, 2001).

2. Materials and methods

2.1. Animals

Larval sea lampreys (*Petromyzon marinus*) collected in May from the Harris River in Port Perry, Ontario, were transported to the University of Toronto at Scarborough and housed in fiberglass aquaria at seasonal water temperatures. Sea lampreys from this population that did not metamorphose in the summer months, but were greater than 120 mm in length, were used in all *in vitro* experiments, except those conducted for light microscopy.

Experiments conducted for light microscopy were performed on larval American brook lampreys, *Lampetra appendix*, because sufficient numbers of sea lampreys were not available. American brook lampreys greater than 130 mm in length were collected in the fall from Duffins Creek in Ajax, Ontario, and housed as described above. It has been shown that KClO_4 , also depresses serum TH concentrations and induces metamorphosis in *L. appendix* (Holmes et al., 1999).

2.2. *In vitro* experimental protocol

Five larval lampreys were anesthetized in 0.025% tricaine methanesulfonate (MS-222, Syndel Laboratories, Vancouver, British Columbia, Canada). The endostyle (subpharyngeal gland) from each larva was removed with minimal amounts of surrounding connective tissue. To simplify tissue processing, endostyles used for light microscopy were removed with the ventral body wall attached. Each endostyle was rinsed in ice-cold incubation buffer (IB) prepared as in Ito et al. (1988): 110 mM NaCl, 1.9 mM KCl, 5 mM NaHCO_3 , 10 mM Hepes, 5 mM glucose, 1.1 mM CaCl_2 , 0.6 mM MgCl_2 , 0.2% bovine serum albumin, 0.006% penicillin G, and 0.01% streptomycin sulfate, pH 7.4. After rinsing, endostyles were drained on a Kimwipe, weighed, and placed in a 25 ml Erlenmeyer flask containing 3 ml ice-cold IB.

The *in vitro* experimental protocol used in this study was adapted from the protocol used by Ito et al. (1988) for the incubation of lamprey liver slices. In brief, 25 ml flasks containing five endostyles (4–6 mg each) and 3 ml IB were sealed with a rubber stopper containing an inlet and outlet made from syringe needles (16 gauge) to allow for continuous flushing with a 95% O_2 :5% CO_2 gas mixture during the incubation. The flasks were placed in a reciprocal water bath shaker at 25 °C and shaken at 70 strokes per minute. Following a 15 min equilibration period 0.3, 3, or 30 μCi of carrier-free Na^{125}I (NEN Life Science products, Boston, MA, USA) was added to the flask. The incubation was carried out for 4 h either in the presence (treated) or absence (untreated control) of KClO_4 (Table 1). A low (L- KClO_4 , 0.72 mM) or high (H- KClO_4 , 3.6 mM) dose of KClO_4 was added to the appropriate incubation flasks immediately prior to the addition of Na^{125}I .

After the 4 h incubation period, endostyles were washed for 45 min in seven to ten changes of IB containing 10 mM KI (to prevent further incorporation of $^{125}\text{I}^-$). Pilot experiments indicated that this washing protocol removed most of the unincorporated $^{125}\text{I}^-$, since the gamma radiation emitted by the wash buffer from the final two changes was comparable to background levels. The gamma emission rate of each endostyle was then measured in counts per minute (cpm)

Table 1

Radioiodide (Na^{125}I) doses and potassium perchlorate (KClO_4) treatment concentrations administered to larval sea lamprey (*Petromyzon marinus*) endostyles in vitro and the data collected from the endostyles in experiments 1–5

Experiment	Na^{125}I (μCi)	KClO_4 (mM)	Data collected ^a
1	3	0.72	IU, IO, and Tg content
2	30	0.72	IU, IO, and Tg content
3	3	0.72	IU, IO, and Tg content
	3	3.6	IU, IO, and Tg content
4	30	0.72	IU, IO, and Tg content
	30	3.6	IU, IO, and Tg content
5	0.3	0.72	IU

^a IU, iodide uptake; IO, iodide incorporation (organification) into Tg.

using a Beckman 4000 gamma counter to determine the amount of $^{125}\text{I}^-$ taken up by the endostyle. For each experiment, the mean cpm of KClO_4 -treated endostyles was expressed as the percentage of the mean of untreated control endostyles. Subsequent to gamma counting, endostyles were either fixed in Bouin's fluid for light microscopy or flash-frozen in liquid nitrogen and stored at -86°C for electrophoretic analysis.

2.3. Light microscopy

Endostyles incubated with $3\mu\text{Ci}$ of Na^{125}I in the presence and absence of L-KClO_4 were fixed in Bouin's fluid for 24 h, dehydrated in a graded series of ethanols, cleared in Histo-Clear (DiaMed Lab Supplies, Mississauga, Ontario, Canada), and embedded in Tissue-Prep (Fisher Scientific, Whitby, Ontario, Canada). Tissue was serially sectioned ($6\mu\text{m}$) and mounted on chromic-sulfuric acid cleaned slides (Chromerge, VWR Scientific, Mississauga, Ontario, Canada). Tissue sections used for light microscopic autoradiography (LM-autoradiography) were hydrated, oxidized in 0.5% aqueous periodic acid, rinsed in distilled water, air-dried, and dip-coated with Kodak NTB-2 nuclear track emulsion (Kodak, Rochester, NY, USA) (Kopriwa and Leblond, 1962). The emulsion was exposed at -20°C for 7–14 days, developed in Kodak D-19 Developer, rinsed in water, and fixed in Kodak Fixer. Developed slides were stained with Lillie's "cold" Schiff reagent (Sheehan and Hrapchak, 1980), counterstained with Mayer's acid haemalum (modified by Lillie, 1942) and 2% aqueous orange G, and mounted with Pro-Texx mounting media (Baxter Diagnostics, Deerfield, IL, USA). Experimental tissue sections that were not dip-coated were processed as above, except that these slides were transferred from the distilled water rinse following oxidation directly into Lillie's "cold" Schiff reagent.

Several endostyles were also fixed immediately after excision and prepared for light microscopy as described above. These unincubated control endostyles were used

for comparison with incubated endostyles to determine if the 4 h incubations had any adverse effects on endostyle morphology and cellular structure. In addition, some unincubated control endostyles were excised in the same manner as the endostyles homogenized for electrophoretic studies (i.e., without the ventral body wall attached) and processed for light microscopy. This procedure was carried out to ensure that the endostyles used for electrophoretic analysis were intact and contained minimal extraneous tissue.

2.4. Electrophoresis

Protein samples were prepared by homogenizing five endostyles in 1 ml lysis buffer (20 mM Tris-HCl [pH 7.5], 300 mM NaCl, 1% Igepal CA-630 [Nonidet P-40], 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, and 57 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged at $13,000g$ for 30 min at 4°C to remove particulate matter and the supernatant was collected. The protein concentration of each sample was determined using the Bradford protein assay (Bradford, 1976). Samples were aliquoted and stored at -86°C until they were used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Samples were prepared for electrophoresis by mixing an equal volume of the protein sample with sample buffer (100 mM Tris-HCl, 200 mM dithiothreitol, 20% glycerol, 4% SDS, and 0.002% bromophenol blue) and boiling for 3 min. When necessary, protein samples were diluted to ensure that the total amount of protein (10 or $15\mu\text{g}$) and the volume (40 or $50\mu\text{l}$) loaded into a given well were the same for all samples.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) using 5% stacking and 7.5% resolving gels. In addition to the endostyle homogenates, broad-range molecular weight markers (Sigma, St. Louis, MO, USA) and 0.1 or $10\mu\text{g}$ porcine Tg (Sigma) were loaded onto each gel. The porcine Tg used was electrophoretically heterogeneous, consisting of numerous fragments of porcine Tg likely of endogenous proteolytic origin, as supplied by the manufacturer. Further purification of this porcine Tg was not performed. Following electrophoresis, gels were processed either for gel-autoradiography to determine the amount of $^{125}\text{I}^-$ incorporated into thyroglobulin or for Western blotting to determine the amount of thyroglobulin in KClO_4 -treated endostyles relative to untreated controls. All protein samples from an individual in vitro experiment were always run on the same gel. This process was repeated in quadruplicate for gel-autoradiography and in duplicate for Western blotting for in vitro experiments 1–4 (Table 1).

Gels processed for autoradiography were stained with Coomassie brilliant blue G-250, destained, dried onto filter paper, and exposed to X-ray film (X-OMAT

AR; Kodak) at -86°C for 6–72 h. Gels processed for Western blotting were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) for 1 h and the proteins were electrophoretically transferred to nitrocellulose (Towbin et al., 1979). Nitrocellulose blots were stained with Ponceau S to qualitatively assess equal loading and transfer efficiency, destained in distilled water, and blocked overnight in a solution of 5% dried skim milk in Tween–Tris-buffered saline (10 mM Tris–HCl, 250 mM NaCl, and 0.05% Tween 20, pH 7.5). Blots were incubated for 1 h in a 1:1000 dilution of the IgG fraction of a rabbit anti-human Tg serum (A 0251; Dako Diagnostics, Mississauga, Ontario, Canada), followed by a 1 h incubation in a 1:1000 dilution of a horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG, Amersham-Pharmacia, Piscataway, NJ, USA). Immunoreactive bands were visualized on X-ray emulsion film (Dupont Cronex MRF34; Marconi Medical Systems, Brampton, Ontario, Canada) using a chemiluminescence kit (ECL; Amersham-Pharmacia, Piscataway, NJ, USA) according to manufacturer's instructions.

Molecular mass was estimated by plotting the relative mobility (R_f) of each Sigma molecular weight marker versus the $\log_{10}$ of its molecular mass. The resultant graph produced a linear standard curve. The equation of this standard curve was determined using Cricket Graph III for the Macintosh and used to estimate the molecular mass of lamprey Tg and the two porcine Tg bands based on their R_f values. Molecular masses presented are the average value of the estimates obtained from seven different gels.

2.5. Densitometry and statistical analysis

Densitometry was performed on gel-autoradiographs and Western blot films using a BioRad Imaging Densitometer (Model GS 700) and Multi-Analyst software (Version 1.1, BioRad, Mississauga, Ontario, Canada). Individual bands on a single piece of film were selected and adjusted for differences in background and the derived value was expressed as the adjusted optical density $\times \text{mm}^2$ (OD). The OD of bands from KClO_4 -treated endostyles were expressed as a percentage of the OD of the appropriate untreated controls. This process was repeated a total of eight times, using two different pieces of film, from each gel. The mean values ($\pm 2\text{SE}$) from all replicate gels from each in vitro experiment and the overall mean ($\pm 2\text{SE}$) of the four in vitro experiments are presented (Fig. 5).

Analysis of variance (ANOVA; blocks design) was used to test for statistically significant differences in the gamma emission rates of endostyles between experimental groups (i.e., control, L- KClO_4 , and H- KClO_4). Each of the five in vitro experiments represented a block in the ANOVA, which was designed to correct the means

for variation between blocks. Densitometric data from autoradiographs and Western blot films were analyzed for statistically significant differences using a pairwise Student's t test. Densitometric data from H- KClO_4 and L- KClO_4 experimental groups were pooled into a single KClO_4 treatment group for statistical comparison with controls. Statistical analyses were performed using "The SAS System, v. 8.0" software package, (SAS Institute, Cary, NC). Differences between means were accepted as statistically significant if $P < 0.05$.

3. Results

Following a 4 h in vitro incubation with Na^{125}I , in the presence (data not shown) and absence of KClO_4 , sections of *L. appendix* endostyles examined by light microscopy showed no morphological differences from endostyles fixed immediately after excision. The organization of the anterior chambers, posterior medial chamber, posterior lateral chambers, and duct opening to the pharynx was similar to previous descriptions (for review see Barrington and Sage, 1972). As depicted in Figs. 1A and C, the epithelial cells lining the endostyle lumen were similar in morphology to those previously described and, thus, showed no visible effects from the incubation period. Routine light microscopy also confirmed that dissection, as performed for the electrophoretic studies, consistently removed an entirely intact endostyle with little accompanying extraneous tissue.

Silver grains were primarily localized over the type 2c and 3 endostylar cells (Figs. 1B and D), indicating that these were the primary cell types involved in the uptake and incorporation of $^{125}\text{I}^-$ in vitro. Type 2c and 3 cells are also the primary iodide-binding cell types in sea lampreys, in vivo (Wright and Youson, 1976). Although some grains are visible above the type 1 cells of the glandular tracts and the connective tissue surrounding the endostyle (Figs. 1B and D), these silver grains were considered background because similar levels were visible in regions of the slide lacking tissue sections as well as on non-radiolabelled control slides (tissue sections from unincubated control endostyles; data not shown).

The uptake of $^{125}\text{I}^-$ by the larval lamprey endostyle in vitro was determined by measuring the gamma radiation emission rates of endostyles, following a 4 h incubation with 0.3, 3, or 30 μCi of Na^{125}I . The levels of gamma radiation emitted by endostyles incubated in the presence of either L- KClO_4 or H- KClO_4 were significantly ($P = 0.03$) lower than those of untreated control endostyles; significant differences between the L- KClO_4 and H- KClO_4 treatment groups were not observed. Overall, L- KClO_4 - and H- KClO_4 -treated endostyles emitted only 47 ± 20 and $39 \pm 7\%$, respectively, of the radioactivity emitted by untreated controls (Fig. 2). Thus, KClO_4 inhibited the uptake of $^{125}\text{I}^-$ by the lamprey

Fig. 2. The percentage of $^{125}\text{I}^-$ uptake, as determined by gamma radiation emissions, by potassium perchlorate (KClO_4) treated endostyles relative to untreated (control) endostyles following a 4 h in vitro incubation with Na^{125}I . Endostyles were incubated with either 0.3 μCi (experiment 5), 3.0 μCi (experiments 1 and 3) or 30 μCi (experiments 2 and 4) of Na^{125}I . KClO_4 treatment concentrations were either 0.72 mM (L- KClO_4) or 3.6 mM (H- KClO_4). Analysis of variance indicated that $^{125}\text{I}^-$ uptake by endostyles in vitro was significantly ($P = 0.03$) reduced following treatment with either L- KClO_4 or H- KClO_4 .

endostyle in vitro. However, L- KClO_4 -treated endostyles in replicate experiment number 4 emitted 86% of the radioactivity emitted by untreated control endostyles (Fig. 2).

Polyacrylamide gel electrophoresis and autoradiography showed that $^{125}\text{I}^-$ taken up by the endostyle in vitro is incorporated into a high molecular mass protein with an electrophoretic mobility similar to reduced porcine Tg (Fig. 3). Western blotting using a rabbit anti-human Tg serum that cross-reacted with porcine Tg detected a single immunoreactive protein with the same electrophoretic mobility as the protein visualized by gel-autoradiography (Fig. 4). These data are consistent with the identification of this protein as a lamprey Tg. Using SDS-PAGE under reducing conditions, the molecular masses of lamprey Tg, the primary porcine Tg band, and a less conspicuous, slower migrating porcine Tg band were estimated to be 226, 212, and 236 kDa, respectively (Fig. 3A).

KClO_4 treatment significantly ($P = 0.01$) reduced the amount of $^{125}\text{I}^-$ incorporated into lamprey Tg by larval lamprey endostyles. Densitometric analysis of gel-autoradiographs indicated that those endostyles incubated in the presence of L- KClO_4 or H- KClO_4 incorporated an average of only $46 \pm 20\%$ (sample size $[N] = 4$) or $58 \pm 42\%$ ($N = 2$), respectively, of the $^{125}\text{I}^-$ incorporated

Fig. 3. (A) Autoradiograph showing radioiodide incorporation and organification into a lamprey thyroglobulin (Tg) by larval lamprey endostyles incubated in vitro with 3 μCi Na^{125}I . Protein samples (10 μg total protein/lane) from untreated (control) endostyles (CON) and endostyles treated with either a low (L- KClO_4 ; 0.72 mM) or high (H- KClO_4 ; 3.6 mM) dose of potassium perchlorate (KClO_4) are shown. The 205 kDa molecular weight marker and a porcine thyroglobulin sample (P-Tg; 10 μg) from the Coomassie blue-stained gel have been pasted on the left and right of the autoradiograph, respectively. The autoradiograph and Coomassie blue-stained lanes are from in vitro experiment 3 (see Table 1). (B) Typical Coomassie blue-stained gel used for autoradiography. This gel shows the protein profile of endostyle homogenates (10 μg total protein/lane) from in vitro experiment 4 (see Table 1), the molecular weight markers (left), and a P-Tg sample (right).

by untreated control endostyles. The percent of $^{125}\text{I}^-$ incorporated by KClO_4 -treated endostyles relative to control endostyles, for each replicate experiment, is presented in Fig. 5A. Overall, the data indicate that KClO_4 significantly inhibits the ability of the lamprey endostyle to organify iodide in vitro. However, in experiments 3 and 4, H- KClO_4 and L- KClO_4 endostyles, respectively, did not show an appreciable decrease in $^{125}\text{I}^-$ incorporation relative to the control endostyles (Fig. 5A).

The total amount of Tg in the larval lamprey endostyle was significantly ($P = 0.01$) reduced relative to

Fig. 4. Immunodetection of thyroglobulin (Tg) in larval lamprey endostyles by Western blot using an IgG purified rabbit anti-human Tg serum, following an in vitro incubation with 30 μ Ci Na^{125}I . Protein samples (10 μ g total protein/lane) from untreated (control) endostyles (CON), endostyles treated in vitro with either a low (L- KClO_4 ; 0.72 mM) or high (H- KClO_4 ; 3.6 mM) dose of potassium perchlorate (KClO_4), and a porcine Tg sample (P-Tg; 0.1 μ g) are shown. The porcine Tg sample used in this Western blot was electrophoretically heterogeneous. The single band shown represents only a portion of the 0.1 μ g porcine Tg loaded (see Section 2).

control values, following a 4 h incubation in the presence of KClO_4 (Fig. 5B). As determined by immunoreactivity with a Tg antiserum and subsequent densitometry, endostyles incubated with Na^{125}I in the presence of L- KClO_4 had $55 \pm 13\%$ of the Tg found in endostyles incubated with only Na^{125}I (Fig. 5B). High- KClO_4 treatment had similar effects on endostylar Tg levels; H- KClO_4 -treated endostyles had Tg levels that were $61 \pm 6\%$ of the levels in untreated controls (Fig. 5B).

4. Discussion

Morphological and biochemical observations have been presented that indicate that the larval lamprey endostyle is viable following a 4 h incubation period in an in vitro experimental system. Endostyles incubated with Na^{125}I in the presence or absence of KClO_4 did not differ in tissue or cellular organization from those endostyles examined immediately after excision. At the level of light microscopy, the morphology of the incubated endostyles (Fig. 1) was comparable to that reported for endostyles that were not incubated in vitro (see Barrington and Sage, 1972). Moreover, the uptake of $^{125}\text{I}^-$ from the incubation medium, its binding to the appropriate cell types, and its incorporation into a Tg-like molecule indicate that the endostyles incubated with Na^{125}I were biologically active. These observations were taken as evidence that endostyles incubated in vitro are performing biochemical processes, associated with TH synthesis, which are similar to those occurring in vivo.

A single iodoprotein was identified in larval lamprey endostyle homogenates. This iodoprotein had an electrophoretic mobility similar to reduced porcine Tg and

Fig. 5. (A) The percentage of $^{125}\text{I}^-$ incorporation (organification) into lamprey thyroglobulin (Tg) by endostyles treated either with a low (L- KClO_4 ; 0.72 mM) or high (H- KClO_4 ; 3.6 mM) dose of potassium perchlorate, relative to untreated (control) endostyles, following a 4 h in vitro incubation with Na^{125}I in four separate experiments. (B) The percentage of thyroglobulin detected by Western blot in L- KClO_4 and H- KClO_4 -treated endostyles, relative to untreated (control) endostyles, following a 4 h in vitro incubation with Na^{125}I . Data were obtained by dividing the adjusted optical densities (OD) of bands from KClO_4 -treated endostyles (pool of five) by those of control endostyles (pool of five), following SDS-polyacrylamide electrophoresis and gel-autoradiography (A) or Western blotting (B). Data presented for gel-autoradiography and Western blotting are the mean values ($\pm 2\text{SE}$) from four and two replicate gels, respectively. Endostyles were incubated with either 3 μ Ci (experiments 1 and 3) or 30 μ Ci (experiments 2 and 4) of Na^{125}I . Pairwise Student's *t* test confirmed that KClO_4 -treated endostyles incorporated significantly ($P = 0.01$) less $^{125}\text{I}^-$ into Tg and contained significantly ($P = 0.01$) less Tg than control endostyles.

was immunoreactive with a polyclonal antibody directed against human Tg (Figs. 3 and 4); thus, this protein was designated as lamprey Tg. Several investigators have identified Tg-like iodoproteins in the larval lamprey endostyle and adult lamprey thyroid tissue, but neither a

definitive size nor sedimentation coefficient has been agreed upon for lamprey Tg. The collection of data from these studies suggests that lampreys possess a combination of iodoproteins and/or Tg subunits similar to those found in higher vertebrates. Included among these iodoproteins are molecules with sedimentation coefficients of 3–8 S, 12 S, and/or 17–19 S (Aloj et al., 1961; Monaco et al., 1978; Roche et al., 1968; Suzuki and Kondo, 1973; Suzuki et al., 1975). Aloj et al. (1967) isolated a native subunit of lamprey Tg with a sedimentation coefficient of 11.7 S and determined its molecular mass to be 331 kDa; this subunit is similar in size to the 12 S (330 kDa) mammalian Tg monomer. Numerous small iodoproteins are also found in mammalian thyroid glands. In mammals, these smaller iodoproteins are formed when Tg is cleaved during the process of iodination; this endogenous cleavage is likely a normal step in the production of TH (Dunn et al., 1983; Ekholm, 1990).

In the present work, the molecular mass of lamprey Tg was estimated to be 226 kDa and that of porcine Tg was 212–236 kDa as determined by SDS-PAGE under reducing conditions. These molecular mass estimates are lower than expected and may be a result of either Tg cleavage or proteolytic activity, as discussed above, or they may be related to the anomalous electrophoretic mobility of large glycoproteins. There was no evidence of any small iodoproteins or protein fragmentation in the gel autoradiographs or Western blots, but with the electrophoretic conditions we employed, the separation of proteins or protein fragments smaller than 25 kDa was not possible.

In a follicular thyroid gland, KClO_4 acts as a competitive inhibitor of iodide uptake by follicle cells (Wolff and Maurey, 1963). The perchlorate ion (ClO_4^-) and similar anions compete with iodide for active transport by an iodide pump or transporter located in the basolateral membrane of the follicle cells (Ekholm, 1990; Gentile et al., 1995). The mode of action of these anionic competitive inhibitors differs from those of other goitrogens such as the thioureyne drugs (thiourea, thiouracil, propylthiouracil, and methimazole), which inhibit thyroid peroxidase-catalyzed iodination of Tg (Gentile et al., 1995). The data provided in the present study indicate that the goitrogen KClO_4 acts directly on the larval lamprey endostyle to inhibit thyroidal activity. Relative to control endostyles, those incubated in the presence of KClO_4 contained significantly less radioactive iodide (Fig. 2), incorporated significantly less iodide into Tg (Fig. 5A), and had significantly less Tg (Fig. 5B). Significant differences in the gamma emission rate, $^{125}\text{I}^-$ incorporation into Tg, and total Tg between L- KClO_4 and H- KClO_4 -treated endostyles were not observed. Similarly, the efficacy of L- KClO_4 and H- KClO_4 treatments at lowering serum TH titers did not differ in larval sea lampreys treated in vivo (Youson et al., 1995).

Western blotting showed that KClO_4 administered to larval lamprey endostyles in vitro lowered the total amount of Tg detected in endostyle homogenates. Endostyles incubated in the presence of KClO_4 had 55–67% of the total Tg measured in control endostyles (Fig. 5B). This level was similar to the decrease in iodide incorporation measured in response to KClO_4 treatment (46–58% of untreated controls; Fig. 5A). Unfortunately, the porcine Tg sample we used was electrophoretically heterogeneous and numerous fragments of porcine Tg were immunoreactive with the antibody. Due to this heterogeneity, we could not use the porcine Tg as a standard with Western blotting to estimate the amount of lamprey Tg per milligram of endostyle protein for comparison with other species. Western blotting data were used only to make relative comparisons between control and KClO_4 -treated endostyles.

The data collected from these in vitro experiments could be interpreted in several ways, depending on whether KClO_4 affects the total Tg content in the larval endostyle directly or indirectly. The decrease in iodide organification in the presence of KClO_4 is likely a result of the inhibition of iodide uptake and the resultant absence of sufficient cellular iodide. This decrease in iodide organification may subsequently result in a decrease in total endostylar thyroglobulin either by decreased synthesis, increased secretion or increased degradation (i.e., reduced stability). Alternatively, the decrease in iodide incorporation may be related to the observed decrease in total endostylar Tg. A decrease in endostylar Tg may be a result of either the direct effects that KClO_4 has on Tg synthesis or secretion, or be a consequence of a general toxic effect of KClO_4 on the endostyle during a 4 h in vitro incubation. Unfortunately, further studies are necessary to provide the definitive description of how KClO_4 reduces thyroidal activity by endostyles in vitro; however, the data are sufficient to permit some speculation.

The observed decrease in total Tg detected in KClO_4 -treated larval endostyles, relative to controls, may be a result of the direct effects of KClO_4 on Tg synthesis or secretion and be independent of the effects that it has on iodide uptake. If this hypothesis holds true, then the results of the current study indicate that a decrease in iodide incorporation into Tg in response to KClO_4 -treatment is due to the fact that KClO_4 -treated endostyles were either synthesizing less, or secreting more, Tg than control endostyles. Alternatively, the decreases in iodide incorporation into Tg and/or the total amount of Tg may be secondary consequences of the effects that KClO_4 has on iodide uptake. This latter idea is more consistent with the known mode of action of KClO_4 on the thyroid gland in other vertebrates (i.e., inhibition of iodide uptake; Wolff and Maurey, 1963). A reduction in the uptake of iodide eventually decreases the amount of cellular iodide available for Tg iodination, resulting in an increase in the proportion of uniodinated or poorly

iodinated Tg. In the larval endostyle, higher cellular concentrations of poorly iodinated Tg may in turn either decrease the rate of Tg synthesis or increase secretion and degradation rates, ultimately decreasing the amount of Tg in the endostyle. However, the notion that KClO_4 produces a decrease in Tg synthesis is contradictory to what is observed in the thyroid systems of other vertebrates (Wolff and Maurey, 1963).

In general, the treatment of vertebrates with a goitrogen results in the inhibition of Tg iodination, either by inhibiting iodide uptake or the iodination reaction. This inhibition prevents the synthesis of TH and lowers serum TH concentrations (Cooper, 1990; Gentile et al., 1995). Decreases in serum TH concentrations feed back to the hypothalamic–pituitary axis and trigger the release of thyrotropin (TSH; thyroid stimulating hormone) (Wilber, 1995). Thyrotropin stimulates a number of factors involved in the synthesis and secretion of TH, including Tg synthesis. In the presence of a goitrogen, the iodination of Tg is prevented and large amounts of poorly iodinated Tg can accumulate in the follicular thyroid gland (Cooper, 1990). This feedback system does not apply to this study for several reasons: (i) the larval lamprey endostyle does not have the ability to store Tg in the same manner as follicular thyroid tissue; therefore, the regulation of Tg synthesis and secretion in the endostyle likely differs from that of a follicular thyroid gland; (ii) in this *in vitro* study, the endostyle was isolated from any hypothalamic–pituitary influences; (iii) there is no evidence in this or any other study to suggest that the larval lamprey endostyle is regulated by the hypothalamic–pituitary axis. Thus, it is conceivable that a goitrogen such as KClO_4 could depress Tg synthesis through its effects on iodide uptake.

In summary, we have tested an *in vitro* experimental system that will be a useful tool in studying both TH synthesis by the larval lamprey endostyle and the factors involved in its regulation. At the same time, the present study has shown with this new tool that the goitrogen KClO_4 acts directly on the larval lamprey endostyle to inhibit thyroïdal activity. This action was evidenced by decreases in the uptake and organification of iodide by the endostyle and the total amount of Tg in this organ.

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