Calcium and calmodulin in the regulation of human thyroid adenylate cyclase activity

Trevor LAKEY,* Sheila MAC NEIL,* Hazel HUMPHRIES,* Simon W. WALKER,†
Donald S. MUNRO* and Stephen TOMLINSON*

*Department of Medicine, Clinical Sciences Centre, Northern General Hospital, Herries Road, Sheffield S5 7AU, U.K., and †Department of Chemical Pathology, Royal Hallamshire Hospital, Sheffield S10 2RX, U.K.

(Received 19 June 1984/Accepted 17 October 1984)

1. TSH (thyrotropin)-stimulated human thyroid adenylate cyclase has a biphasic response to Ca^{2+} , being activated by submicromolar Ca^{2+} (optimum 22nM), with inhibition at higher concentrations. 2. Calmodulin antagonists caused an inhibition of TSH-stimulated adenylate cyclase in a dose-dependent manner. Inhibition of TSH- and TSIg-(thyroid-stimulating immunoglobulins)-stimulated activity was more marked than that of basal, NaF- or forskolin-stimulated activity. This inhibition was not due to a decreased binding of TSH to its receptor. 3. Addition of pure calmodulin to particulate preparations of human non-toxic goitre which had not been calmodulin-depleted had no effect on adenylate cyclase activity. 4. EGTA was ineffective in removing calmodulin from particulate preparations, but treatment with the trivalent metal ion La^{3+} resulted in a loss of up to 98% of calmodulin activity from these preparations. 5. Addition of La^{3+} directly to the adenylate cyclase assay resulted in a partial inhibition of TSH- and NaF-stimulated activity, with 50% inhibition produced by 5.1 μM and 4.0 μM - La^{3+} respectively. 6. Particulate preparations with La^{3+} showed a decrease of TSH- and NaF-stimulated adenylate cyclase activity (approx. 40–60%). 7. In La^{3+} -treated preparations there was a decrease in sensitivity of TSH-stimulated adenylate cyclase to Ca^{2+} over a wide range of Ca^{2+} concentrations, but most markedly in the region of the optimal stimulatory Ca^{2+} concentration. 8. In particulate preparations from which endogenous calmodulin had been removed by La^{3+} treatment, the addition of pure calmodulin caused an increase ($73 \pm 22\%$; mean $\pm$ S.E.M., $n = 8$) in TSH-stimulated thyroid adenylate cyclase activity. This was seen in 8 out of 13 experiments.

It is now recognized that calmodulin plays a major role in the mediation of the effects of Ca^{2+} on cellular function (Brostrom & Wolff, 1981). Calmodulin is involved in the regulation of many cell processes, including cyclic nucleotide metabolism. There is good evidence for the presence of calmodulin-activated cyclic nucleotide phosphodiesterases in a wide variety of tissues (Wang & Weisman, 1979). However, by comparison the effects of calmodulin on adenylate cyclase have been studied in relatively few tissues (Tomlinson *et al.*, 1984), although many adenylate cyclase sys-

tems have been shown to be Ca^{2+} -sensitive (Bradham & Cheung, 1980).

The potential importance of Ca^{2+} in the regulation of thyroid cell function is suggested by the Ca^{2+} requirement of a number of important thyroid cell functions (Willems *et al.*, 1971). We have previously purified calmodulin from human thyroid and have reported that TSH-stimulated cyclic AMP accumulation in cultured human thyroid cells can be inhibited by calmodulin antagonists (Ollis *et al.*, 1983).

In the present paper we examine the Ca^{2+} -sensitivity of human thyroid adenylate cyclase and investigate the involvement of calmodulin in this sensitivity.

Abbreviations used: TSH, thyrotropin; TSIg, thyroid-stimulating immunoglobulins.

Materials and methods

[α - 32 P]ATP (0.5–30 Ci/mmol), cyclic [8- 3 H]-AMP (20–30 Ci/mmol), 45 CaCl₂ and the calmodulin radioimmunoassay kit were obtained from Amersham International, Amersham, Bucks., U.K. Cyclic AMP as the crystallized free acid, the sodium salt of ATP, phosphoenolpyruvate (tricyclohexylammonium salt), pyruvate kinase (EC 2.7.1.40; sp. activity approx. 200 units/mg) and calmodulin-deficient bovine heart phosphodiesterase were obtained from Boehringer Mannheim, London, U.K. Snake venom (*Ophiophagus hannah*), Dowex 1-X8 (200–400 mesh) anion-exchange resin, Coomassie Blue, LaCl₃, bovine serum albumin (fraction V), isobutylmethylxanthine, guanylylimidodiphosphate, Chelex resin, TSH (bovine pituitary; 1 unit/mg) and theophylline were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Forskolin was obtained from Calbiochem/Behring Corp., La Jolla, CA, U.S.A. Tri-fluoperazine, prochlorperazine and prochlorperazine sulphoxide were gifts from Smith, Kline and French Laboratories, Welwyn Garden City, Herts., U.K. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulphonamide (W7) was obtained from Rikaken Co., 23-8 Kosaka, Inokoishi, Idaka-cho, Meito-ku, Nagoya 465, Japan.

All other chemicals were of analytical grade.

Measurement of adenylate cyclase activity in human non-toxic-goitre particulate preparations

Human thyroid adenylate cyclase was prepared from 12 non-toxic goitres obtained at surgery. Tissue specimens were processed at 4°C as soon as possible after removal (usually within 60 min). Tissue was finely minced with scalpel blades and suspended in approx. 10 vol. of 25 mM-Tris/HCl (pH 7.4 at 4°C)/250 mM-sucrose (preparation buffer). This suspension was then homogenized with five strokes of a loose Dounce glass pestle homogenizer, and the homogenate was filtered through muslin. Homogenates were centrifuged at 7500g (r_{av} . 6.0 cm) in a Sorvall RC5B centrifuge for 10 min at 4°C to yield a particulate preparation, which was washed twice (centrifugation as before) before storage at –80°C at 5 mM-Mes (pH 6.5) containing 30% (v/v) ethylene glycol, 1 mM-dithiothreitol, 5% (w/v) glucose, 50 mM-NaCl and 50 mg of phenylmethanesulphonyl fluoride/litre of buffer (storage buffer). Before use in each assay, samples of particulate preparation were washed once with preparation buffer.

In some experiments preparations were divided, and part of each was preincubated with preparation buffer containing LaCl₃ (at the concentrations shown) for 15 min at 4°C before washing twice with La³⁺-free preparation buffer. Control

portions of each preparation were handled similarly in La³⁺-free preparation buffer.

Adenylate cyclase activity was measured by the conversion of [32 P]ATP into cyclic [32 P]AMP in particulate preparations of human non-toxic goitre as described previously (Mac Neil *et al.*, 1980). Reaction mixtures (100 μ l) contained 25 mM-Tris/HCl (pH 7.8), 1 mM-[α - 32 P]ATP (20–200 d.p.m./pmol), 1 mM-cyclic AMP, 2.5 mM-phosphoenolpyruvate, 1.5 units of pyruvate kinase, 130 μ g of bovine serum albumin/ml, 30 mM-KCl and 4.5 mM-MgSO₄. Incubations were for 10 min at 37°C. Cyclic [32 P]AMP was isolated as described by Salomon *et al.* (1974). Protein was determined by the method of Bradford (1976).

Preparation of calmodulin

Pig brain and eel electric-organ calmodulin were prepared by the method of Kakiuchi *et al.* (1981). Preparations were over 95% pure as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970).

Phosphodiesterase assay and measurement of calmodulin

Measurement of calmodulin activity by using a calmodulin-responsive bovine heart phosphodiesterase was by the method described previously (Mac Neil *et al.*, 1984). Incubations (final volume 400 μ l) contained 40 mM-Tris/HCl (pH 7.0 at 37°C), 4 mM-2-mercaptoethanol, 5 mM-MgCl₂, cyclic [3 H]-AMP (2×10^5 c.p.m./tube), 100 μ M-cyclic AMP, 25 μ M-CaCl₂ and calmodulin or calmodulin-containing heat-treated sample as required. Incubations were for 15 min at 37°C. In this assay the endogenous Ca²⁺ present in buffers and calmodulin stock was removed by passage over a column of Chelex resin (as indicated in the text). The efficiency of the Ca²⁺ removal was checked with 45 Ca²⁺, and was greater than 95%.

TSH binding

The TSH radioreceptor assay using human thyroid particulate preparations was as described previously (Humphries *et al.*, 1982).

TSIg preparation

TSIg preparations were obtained by (NH₄)₂SO₄ precipitation of serum samples from thyrotoxic patients (Humphries *et al.*, 1982).

Free Ca²⁺ calculation

For the adenylate cyclase assay, the free Ca²⁺ concentration, in the presence of EGTA, was calculated by using the dissociation constants of Caldwell (1970).

Ca²⁺ measurement in assay buffers

Contaminating Ca²⁺ was measured by atomic absorption spectroscopy.

Results

Ca²⁺-sensitivity of thyroid adenylate cyclase

In human thyroid particulate preparations we have demonstrated that TSH-stimulated adenylate cyclase showed a biphasic response to Ca²⁺ (Fig. 1). This activity was optimally stimulated by a calculated free Ca²⁺ concentration of 21.7 ± 6.8 nM (mean $\pm$ S.E.M., $n = 6$). Maximal TSH-stimulated adenylate cyclase activity was $68 \pm 12\%$ (mean

Fig. 1. *Ca²⁺-dependence of TSH-stimulated adenylate cyclase activity*

Adenylate cyclase activity was measured in washed particulate preparations of three human non-toxic goitres. A maximally stimulating concentration of TSH (200munits/ml) was present at all points shown. TSH-stimulated activity at the lowest Ca²⁺ concentration present (0.5 nM) was designated 100% for each preparation to allow comparison of data. Free Ca²⁺ concentration was calculated from the EGTA and Ca²⁺ dissociation constants of Caldwell (1970). The actual concentrations of Ca²⁺ and EGTA added to the assay to give these free calcium concentrations are shown on the lower abscissa. Values shown are the means $\pm$ S.E.M. of triplicate determinations in three experiments.

$\pm$ S.E.M., $n = 6$; non-toxic-goitre preparation) greater than at the lowest free Ca²⁺ concentration tested (0.5 nM). At higher free Ca²⁺ concentrations the TSH-stimulated adenylate cyclase activity was strongly inhibited. For example, at 0.2 μ M free Ca²⁺, activity was $18.6 \pm 3.2\%$ (mean $\pm$ S.E.M., $n = 6$) of the activity at optimal free Ca²⁺.

Effect of calmodulin antagonists on adenylate cyclase activity

Preincubation of human thyroid particulate preparations with calmodulin antagonists (10 min at 4°C before assay) inhibited adenylate cyclase activity, the effect being most marked on TSIg- and TSH-stimulated enzyme activity, as shown in the experiment depicted in Fig. 2 for the calmodulin antagonist W7. The concentration of W7 required to produce half-maximal inhibition of enzyme activity, based on three non-toxic-goitre preparations, was 3 ± 1 μ M for TSIg (10mg/ml), 91 ± 30 μ M for TSH (200munits/ml), 210 ± 37 μ M for forskolin (0.1 mM), 303 ± 73 μ M for basal and 370 ± 96 μ M for NaF (10mM)-stimulated enzyme activity (means $\pm$ S.D., $n = 3$). Prochlorperazine produced half-maximal inhibition of TSH

Fig. 2. *Inhibitory effect of calmodulin antagonist W7 on thyroid adenylate cyclase activity*

Adenylate cyclase activity was measured as described in the Materials and methods section. Results shown are means $\pm$ S.E.M. of triplicate determinations in a single representative experiment in which a non-toxic-goitre preparation was preincubated with W7 for 10 min at 4°C (at which time the inhibitory potency of the drug was maximal) and then enzyme activity was measured in the presence of buffer alone ($\blacktriangle$), 10mg/ml TSIg ($\square$), 200munits of TSH/ml ($\bullet$), 0.1 mM-forskolin ($\circ$) or 10mM-NaF ($\blacksquare$). The concentrations of W7 required to produce half-maximal inhibition of enzyme activity based on three such preparations are given in the text.

(200 munits/ml)-stimulated enzyme activity at $47 \pm 6.2 \mu\text{M}$ (mean $\pm$ S.D., $n = 3$), whereas the sulphoxide analogue of prochlorperazine was ineffective in inhibiting TSH-stimulated enzyme activity at the highest concentration tested ($200 \mu\text{M}$). All experiments with calmodulin antagonists were performed in the absence of EGTA without addition of further Ca^{2+} .

There was no evidence that calmodulin antagonists inhibited the binding of TSH to human thyroid when assessed by radioreceptor assay techniques: incubation of human thyroid membranes, prepared from thyrotoxic tissue, with concentrations of prochlorperazine, its sulphoxide or W7 between 0.07 and $7 \mu\text{M}$ for 1 h at 37°C had no effect on the percentage of ^{125}I -labelled bovine TSH bound, when compared with buffer controls. Moreover, with prochlorperazine and W7 at concentrations of 7 – $70 \mu\text{M}$ there was a dose-dependent enhancement of the binding of ^{125}I -TSH to human thyroid membranes, with an approx. 2-fold increase over buffer controls in the presence of $70 \mu\text{M}$ -prochlorperazine or -W7; prochlorperazine sulphoxide, however, remained ineffective over these concentrations. The calmodulin antagonists did not affect the ability of unlabelled TSH to displace ^{125}I -TSH from particulate preparations.

Effect of calmodulin on untreated thyroid-particulate-preparation adenylate cyclase

In an attempt to demonstrate by direct means the involvement of calmodulin in the activation of human thyroid adenylate cyclase, pure calmodulin (10 ng – $10 \mu\text{g}$ per $100 \mu\text{l}$ of incubation) was added to the adenylate cyclase assay. There was, however, no significant effect of added calmodulin on either basal or TSH-stimulated adenylate cyclase activity.

Removal of calmodulin from thyroid particulate preparations by using La^{3+}

Calmodulin was present in thyroid particulate preparations ($0.45 \pm 0.07 \mu\text{g}/\text{mg}$ of protein), and this accounted for approx. 35% of total thyroid cell calmodulin. Initial attempts to remove this endogenous calmodulin used extensive washing of particulate preparations with EGTA. Up to $20 \mu\text{M}$ -EGTA proved ineffective in removing particulate calmodulin (Table 1). However, incubation of particulate preparations with La^{3+} resulted in a loss of calmodulin activity (Table 1). A decrease in activity of up to 98% was achieved with this technique. Calmodulin activity could be detected in the buffer washes of particulate preparations after La^{3+} treatment by radioimmunoassay for calmodulin.

Effect of La^{3+} on thyroid adenylate cyclase activity

Addition of La^{3+} (0.1 – $100 \mu\text{M}$) directly to the adenylate cyclase assay partially inhibited enzyme activity. At maximal inhibition there was $43 \pm 9\%$ (mean $\pm$ S.E.M., $n = 6$) of TSH-stimulated activity remaining and $30 \pm 4\%$ (mean $\pm$ S.E.M., $n = 5$) of NaF-stimulated activity remaining. However, comparatively low concentrations of La^{3+} produced 50% inhibition of agonist-stimulated enzyme activity, i.e. $5.1 \pm 2.3 \mu\text{M}$ (mean $\pm$ S.E.M., $n = 4$) for TSH-stimulated activity and $4.0 \pm 1.4 \mu\text{M}$ (mean $\pm$ S.E.M., $n = 4$) for NaF-stimulated activity.

Effect of La^{3+} on calmodulin-dependent bovine heart phosphodiesterase activity

Below $10 \mu\text{M}$, La^{3+} was without effect on calmodulin-activated bovine heart phosphodiesterase under standard assay conditions containing maximal Ca^{2+} and calmodulin. Concentrations higher than $10 \mu\text{M}$ - La^{3+} produced progressive

Table 1. *Effect of La^{3+} treatment on calmodulin activity and adenylate cyclase activity in particulate preparations of non-toxic goitre*

Particulate preparations were prepared as described in the Materials and methods section and incubated with up to 20 mM -EGTA or $25 \mu\text{M}$ -, $100 \mu\text{M}$ - or $200 \mu\text{M}$ - LaCl_3 -containing buffer for 15 min at 4°C . The preparations were then washed twice with buffer. Calmodulin activity and adenylate cyclase activity were measured as described in the Materials and methods section. TSH and NaF concentrations were maximal (200 munits/ml and 10 mM respectively). The percentage decreases in calmodulin activity and in adenylate cyclase activity are expressed relative to a buffer-washed control from the same particulate preparation of non-toxic goitre. Results are means $\pm$ S.E.M. of (n) experiments: N.D., none detectable.

	Loss of calmodulin activity (%)	Loss of adenylate cyclase activity (%)		
		Basal	TSH	NaF
EGTA	4.5 ± 0.5 (2)	N.D.	N.D.	N.D.
$25 \mu\text{M}$ - La^{3+}	18 ± 12 (4)	16 ± 11 (9)	46 ± 10 (9)	64 ± 7 (3)
$100 \mu\text{M}$ - La^{3+}	92 ± 4 (5)	7 ± 11 (5)	64 ± 8 (4)	63 ± 4 (4)
$200 \mu\text{M}$ - La^{3+}	98 ± 0.1 (2)	—	—	—

inhibition of enzyme activity. For example, in one experiment $100\text{ }\mu\text{M-La}^{3+}$ inhibited enzyme activity by 65%. This inhibition could not be overcome by increasing assay Ca^{2+} concentration even to 20 times the concentration of La^{3+} .

La^{3+} on its own, in the absence of Ca^{2+} and calmodulin, had no effect on phosphodiesterase activity below 1 mM . However, in the presence of calmodulin but in the absence of Ca^{2+} , La^{3+} concentrations up to $10\text{ }\mu\text{M}$ progressively stimulated enzyme activity. Higher concentrations progressively failed to maintain this stimulation, as shown in Fig. 3.

Agonist-responsiveness of adenylate cyclase in La^{3+} -treated preparations

In particulate preparations which had been pre-treated with La^{3+} (and then washed twice with La^{3+} -free buffer), there was a decrease in the responsiveness of adenylate cyclase to maximally stimulating concentrations of both TSH and NaF (Table 1) compared with buffer-washed controls from the same non-toxic-goitre preparation. There was comparatively little effect on basal activity (Table 1). The adenylate cyclase activities shown in Table 1 were all measured without manipulation of the assay $[\text{Ca}^{2+}]$. The contaminating Ca^{2+} concentration of the assay buffers was $50\text{ }\mu\text{M}$ (as measured by atomic-absorption spectroscopy).

Fig. 3. Effect of La^{3+} on bovine heart phosphodiesterase activity in the presence of calmodulin

Assay of phosphodiesterase activity was as described in the Materials and methods section. A maximally stimulating concentration of calmodulin was present ($200\text{ ng}/400\text{ }\mu\text{l}$ incubation). Ca^{2+} was removed from all buffers and reagents by passing these through Chelex resin. Basal enzyme activity was $2.1 \pm 0.3\text{ nmol}$ of cyclic AMP hydrolysed/15 min and maximal enzyme activity [in the presence of maximal calmodulin and maximal Ca^{2+} ($25\text{ }\mu\text{M}$)] was $11.9 \pm 0.6\text{ nmol}$ of cyclic AMP hydrolysed/15 min. Values shown are means $\pm$ S.E.M. of triplicate determinations in a single representative experiment.

La^{3+} pre-treatment, although markedly inhibiting the response to TSH, did not significantly affect the amount of TSH required for half-maximal activation of the enzyme [$7.1 \pm 1.6\text{ munits/ml}$ ($n = 5$) pre-treatment and $3.7 \pm 2.7\text{ munits/ml}$ ($n = 3$) post-treatment] as depicted in the example shown in Fig. 4.

Ca^{2+} -sensitivity in La^{3+} -treated preparations

La^{3+} pre-treatment of preparations caused a decrease in sensitivity to Ca^{2+} over a wide range of Ca^{2+} concentration, but most markedly in the region of the optimal Ca^{2+} concentration of the TSH-stimulated enzyme (Fig. 5). Inhibition of enzyme activity by Ca^{2+} was still seen in La^{3+} -treated preparations, and over a similar concentration range to that producing inhibition in untreated preparations. Added calmodulin partially restored the Ca^{2+} -sensitivity of TSH-stimulated adenylate cyclase activity in La^{3+} -treated preparations (Fig. 5).

Fig. 4. Effect of La^{3+} -treatment on TSH-stimulated adenylate cyclase activity

Adenylate cyclase activity was measured as described in the Materials and methods section over a range of TSH concentrations. Values shown are means $\pm$ S.E.M. of triplicate determinations in a single representative experiment in which a non-toxic-goitre preparation was either buffer-washed (control preparation; $\bullet$) or pretreated with La^{3+} ($\triangle$). La^{3+} treatment involved a 15 min preincubation at 4°C with $200\text{ }\mu\text{M-LaCl}_3$, followed by two buffer washes. The calmodulin activity of La^{3+} -treated preparations was 21% of that found in control preparations. The assay was performed at a calculated free Ca^{2+} concentration of 10 nM , based on the dissociation constants of Caldwell (1970).

Fig. 5. Ca^{2+} -sensitivity and calmodulin responsiveness of adenylate cyclase in La^{3+} -treated particulate preparations

Adenylate cyclase activity was measured as described in the Materials and methods section, in the presence of a maximally stimulating concentration of TSH (200 munits/ml). Values shown are means $\pm$ S.E.M. of triplicate determinations in a single representative experiment in which a non-toxic-goitre preparation was either buffer-washed (control preparation; ●) or pretreated with La^{3+} (△, ○). La^{3+} treatment involved a 15 min preincubation at 4°C with 200 μM - LaCl_3 , followed by two buffer washes. The calmodulin activity of control preparations was 325 ng/mg of particulate protein and for La^{3+} -treated preparations was 7 ng/ml of particulate protein. Calmodulin (2 μg /ml) was preincubated with one La^{3+} -treated preparation (○) for approx. 5 min at 4°C before assay; to the other La^{3+} -treated preparation (△) no calmodulin was added. Free Ca^{2+} concentration was calculated from the EGTA and Ca^{2+} dissociation constants of Caldwell (1970). The actual concentrations of Ca^{2+} and EGTA added to the assay to give these free Ca^{2+} concentrations are shown on the lower abscissa. Basal enzyme activity was 0.228 ± 0.061 nmol of cyclic AMP produced/10 min per mg of protein.

Calmodulin responsiveness in La^{3+} -treated preparations

Addition of pure calmodulin in La^{3+} -treated non-toxic-goitre particulate preparations (with a preincubation of approx. 5 min at 4°C before assay) significantly increased TSH-stimulated adenylate cyclase activity in eight out of thirteen experiments. In these experiments the maximal stimulation produced by calmodulin in La^{3+} -treated preparations averaged $73 \pm 22\%$ (mean $\pm$ S.E.M., $n = 8$). In the other five experiments calmodulin

had little or no significant effect on adenylate cyclase activity. As shown in Fig. 6, however, and to some extent in Fig. 5 also, small changes in the free Ca^{2+} concentration present greatly affected the response to calmodulin. This is shown particularly in Fig. 6, where at a free Ca^{2+} concentration of 1 nM calmodulin addition increased TSH-stimulated adenylate cyclase activity, but at a free Ca^{2+} concentration of 4.5 nM it was without effect.

Discussion

The importance of Ca^{2+} in the control of thyroid processes had been increasingly recognized, for example in TSH stimulation of glucose oxidation and in iodide binding to proteins (Willems *et al.*, 1971). Also in the regulation of thyroid adenylate cyclase activity, Ca^{2+} has been shown to have a stimulatory effect on enzyme activity in horse thyroid membranes pretreated with EGTA (Desmedt & Dumont, 1977).

We have shown in the present paper that TSH-stimulated adenylate cyclase activity in human thyroid has a biphasic response to Ca^{2+} , being activated by submicromolar free Ca^{2+} concentrations and inhibited by higher concentrations. This response to Ca^{2+} may be of physiological relevance, since TSH has been found to cause the release of diffusable Ca^{2+} from thyroid slices (Rodesch *et al.*, 1974), and this may be associated with an increase in the intracellular free Ca^{2+} concentration (Rodesch *et al.*, 1976).

The free Ca^{2+} concentration that we have found to be optimal for TSH-stimulated enzyme activity, 22 nM, is of a similar order to that quoted for several other systems. Thus the optimal free Ca^{2+} concentration for adenylate cyclase activity in GH₃ pituitary-tumour cells is 40 nM (Brostrom *et al.*, 1982). However, although most groups state that submicromolar free Ca^{2+} is necessary for optimal activation of adenylate cyclase, some groups quote values in the micromolar range. For example, 8 μM has been found to be optimal for intestinal-epithelial-cell adenylate cyclase (Amiranoff *et al.*, 1983). Almost all groups use various ratios of Ca^{2+} and the Ca^{2+} chelator, EGTA, to manipulate the free Ca^{2+} concentration. Since the free Ca^{2+} concentration may also be affected by such phenomena as the binding of Ca^{2+} to membranes, the large variation in quoted values of optimal free Ca^{2+} may be explained by difficulties in determining the true free Ca^{2+} concentration of the assay system. It is also possible that there are different requirements for Ca^{2+} in different adenylate cyclase systems.

The presence of calmodulin has been demonstrated in human thyroid. In addition, TSH-stimulated cyclic AMP accumulation in cultured

Fig. 6. Effect of calmodulin on TSH-stimulated adenylate cyclase activity in La^{3+} -treated preparations

Adenylate cyclase activity was measured as described in the Materials and methods section in the presence of a maximally stimulating concentration of TSH (200 munits/ml). Non-toxic-goitre preparations were preincubated with $100 \mu\text{M-La}^{3+}$ for 15 min at 4°C , followed by two buffer washes. Calmodulin was preincubated with particulate preparations for approx. 5 min at 4°C , and the assay of adenylate cyclase activity was carried out in incubation buffer containing either 1 nM (a) or 4.5 nM free Ca^{2+} (b). Free Ca^{2+} concentrations were calculated by using the EGTA and Ca^{2+} dissociation constants of Caldwell (1970). Values shown are means $\pm$ S.E.M. of triplicate determinations in a single representative experiment. Statistical significance was tested with the unpaired *t* test. * $P < 0.05$, ** $P < 0.005$.

human thyroid cells is inhibited by calmodulin antagonists (Ollis *et al.*, 1983). These observations, coupled with the increasing number of reports of calmodulin involvement in adenylate cyclase activity in other systems [e.g. guinea-pig enterocyte adenylate cyclase (Pinkus *et al.*, 1983) and rat smooth muscle (Piascik *et al.*, 1983)] led us to investigate whether this protein is involved in thyroid adenylate cyclase activity.

Initial evidence of calmodulin involvement in thyroid adenylate cyclase activity was provided by the results obtained with calmodulin antagonists. Both prochlorperazine and the naphthalene-sulphonamide W7 inhibited TSH-stimulated adenylate cyclase activity in a dose-dependent manner, whereas the relatively inactive calmodulin antagonist prochlorperazine sulphoxide had no significant effect. There was no evidence that this inhibition was due to a decreased binding of TSH to its receptor. Also, the variation in inhibitory potency of W7 between different adenylate cyclase activities with TSIg- and TSH-stimulated activity being more potently inhibited than basal, NaF- or forskolin-stimulated activity argues against these drugs causing a non-specific inhibition of enzyme activity.

There is now increasing evidence that the effects of calmodulin antagonists may not be due solely to specific binding to calmodulin. Vincenzi (1982) has reported that oleate can activate Ca^{2+} -pump ATPase and that the calmodulin antagonist trifluoperazine is capable of abolishing this activation. Other effects of phenothiazines which are not specific to calmodulin include inhibition of Ca^{2+} uptake in pancreatic islets (Henquin, 1981) and decreasing ATP in lymphocytes (Corps *et al.*, 1982). Hence it is desirable that a function for calmodulin should be established by more direct means.

In the presence of endogenous calmodulin, addition of further calmodulin had no effect on thyroid activity. Therefore, in common with other workers, we attempted to deplete membranes of endogenous calmodulin in order to investigate direct activation of adenylate cyclase by added pure calmodulin. The technique most commonly used for calmodulin depletion in particulate preparations is washing with EDTA- or EGTA-containing buffers. The effectiveness of this technique is variable, with 95% removal in brain (Brostrom *et al.*, 1977) and 50% in guinea-pig lung (Ofulue & Nijjar, 1981), but little or no removal in

GH₃ pituitary-tumour cells (Brostrom *et al.*, 1982), guinea-pig spermatozoa (Hyne & Garbers, 1979) or in our present study.

Schultz & Klumpp (1982), studying the involvement of calmodulin in *Paramecium* guanylate cyclase, have reported that La³⁺, a trivalent metal ion, is capable of depleting the ciliary membranes of *Paramecium* of calmodulin. Our own results agree with this finding, using thyroid particulate preparations, where La³⁺ caused a decrease in particulate calmodulin activity and the appearance of radioimmunoassayable calmodulin in particulate washes.

La³⁺ is known to interact with a variety of Ca²⁺-mediated processes (Weiss, 1974). For example, La³⁺ is a potent inhibitor of the Ca²⁺-dependent release of histamine (Foreman & Mongar, 1972). In an investigation into the mechanism of action of La³⁺ we have found (using an assay buffer depleted of Ca²⁺) that La³⁺ can actually stimulate the activity of a calmodulin-dependent bovine heart phosphodiesterase, but only in the presence of calmodulin. Klumpp *et al.* (1983) have previously presented similar findings. This suggests that La³⁺ is binding to the Ca²⁺-binding sites of calmodulin. This is possible, since, as Lettvin *et al.* (1964) have argued, the crystal ionic radii of Ca²⁺ and La³⁺ are approximately similar. However, if La³⁺ can form a complex with calmodulin, it appears in our study and that of Klumpp *et al.* (1983) that the properties of this complex vary among different enzyme systems.

In the present study La³⁺ inhibited, but did not stimulate, adenylate cyclase activity in thyroid particulate preparations. A similar situation was found for the guanylate cyclase of *Paramecium* (Klumpp *et al.*, 1983). The ability of La³⁺ to displace calmodulin from the ciliary membranes of *Paramecium* and particulate preparations of human thyroid may be due to the formation of an inactive La³⁺-calmodulin complex. These differences in the action of La³⁺ on different enzymes suggest that the interaction between calmodulin and its target enzymes may be variable.

Irrespective of the mode of action of La³⁺, it appears to decrease endogenous particulate calmodulin. Associated with this loss, the particulate thyroid adenylate cyclase enzyme shows a marked loss of agonist responsiveness and a decreased Ca²⁺-sensitivity. Adenylate cyclase activity in such calmodulin-depleted membranes can be stimulated by the addition of pure calmodulin, in a dose-dependent manner. The added calmodulin can at least partially restore the Ca²⁺-sensitivity of hormone-stimulated enzyme activity after the decrease in this sensitivity by La³⁺ treatment. It is possible that calmodulin only partially restores Ca²⁺-sensitivity because La³⁺ treatment may

modify the manner of calmodulin interaction with the enzyme.

In conclusion, in this study we have used several approaches, including the direct stimulation of the enzyme by calmodulin, to demonstrate that calmodulin is involved in the regulation of TSH-stimulated human thyroid adenylate cyclase.

This work was supported by a grant from the Medical Research Council to S. M. N. and D. S. M. S. T. is a Wellcome Trust Senior Lecturer. T. L. is an M.R.C. Research Student. We are grateful to Mr. C. H. Talbot, Professor R. G. Clark and Mr. K. T. H. Moore for their help in providing the thyroid tissue used in this study and to Dr. L. Hanford for TSIg preparations.

References

- Amiranoff, B. M., Laburthe, M. C., Rouyer-Fessard, C. M., Demaille, J. & Rosselin, G. E. (1983) *Eur. J. Biochem.* **130**, 33–37
- Brodford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Bradham, L. S. & Cheung, W. Y. (1980) *Calcium Cell Funct.* **1**, 109–125
- Brostrom, C. O. & Wolff, D. J. (1981) *Biochem. Pharmacol.* **30**, 1395–1405
- Brostrom, C. O., Brostrom, M. A. & Wolff, D. J. (1977) *J. Biol. Chem.* **252**, 5677–5685
- Brostrom, M. A., Brotman, L. A. & Brostrom, C. O. (1982) *Biochim. Biophys. Acta* **721**, 227–235
- Caldwell, P. C. (1970) in *Calcium Chelators and Buffers* (Cuthbert, A. W., ed.), pp. 10–16. Macmillan, London
- Corps, A. N., Hesketh, R. & Metcalfe, J. C. (1982) *FEBS Lett.* **138**, 280–283
- Desmedt, D. H. & Dumont, J. E. (1977) *Arch. Int. Physiol. Biochim.* **85**, 403–404
- Foreman, J. C. & Mongar, J. L. (1972) *Nature (London) New Biol.* **240**, 255–256
- Henquin, J. C. (1981) *Biochem. J.* **196**, 771–780
- Humphries, H., Dirmikis, S. & Munro, D. S. (1982) *J. Endocrinol.* **93**, 371–380
- Hyne, R. V. & Garbers, D. L. (1979) *Biol. Reprod.* **21**, 1135–1142
- Kakiuchi, S., Kenji, S., Yamazaki, R., Kambayashi, J., Sakou, M. & Kosaki, G. (1981) *FEBS Lett.* **126**, 203–207
- Klumpp, S., Kleefeld, G. & Schultz, J. E. (1983) *J. Biol. Chem.* **258**, 12455–12458
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lettvin, J. Y., Pickard, W. F. & McCulloch, W. S. (1964) *Nature (London)* **202**, 1338–1339
- Mac Neil, S., Crawford, A., Amirasooli, H., Johnson, S., Pollock, A., Ollis, C. & Tomlinson, S. (1980) *Biochem. J.* **188**, 393–400
- Mac Neil, S., Walker, S. W., Senior, H. J., Bleehen, S. S. & Tomlinson, S. (1984) *J. Invest. Dermatol.* **83**, 15–20
- Ofulue, A. F. & Nijjar, M. S. (1981) *Biochem. J.* **200**, 475–480
- Ollis, C. A., Mac Neil, S., Walker, S. W., Brown, B. L., Sharrard, R. M., Munro, D. S. & Tomlinson, S. (1983) *J. Endocrinol.* **99**, 251–260

- Piascik, M. T., Babich, M. & Rush, M. E. (1983) *J. Biol. Chem.* **258**, 10913–10918
- Pinkus, L. M., Sulimovici, S., Susser, F. I. & Roginsky, M. S. (1983) *Biochim. Biophys. Acta.* **762**, 552–559
- Rodesch, F., Bogaert, C. & Dumont, J. E. (1974) *C. R. Hebd. Seances Acad. Sci. Ser. D.* **278**, 931–934
- Rodesch, F., Bogaert, C. & Dumont, J. E. (1976) *Mol. Cell. Endocrinol.* **5**, 303–313
- Salomon, Y., Landos, C. & Rodbell, M. (1974) *Anal. Biochem.* **58**, 541–548
- Schultz, J. E. & Klumpp, S. (1982) *FEMS Microbiol. Lett.* **13**, 303–306
- Tomlinson, S., Mac Neil, S., Walker, S. W., Ollis, C. A., Merritt, J. E. & Brown, B. L. (1984) *Clin. Sci.* **66**, 497–508
- Vincenzi, F. F. (1982) in *Calmodulin and Intracellular Calcium Receptors* (Kakiuchi, S., Hidaka, H. & Means, A. R., eds.), pp. 1–17, Plenum Press, New York
- Wang, J. K. & Weisman, D. M. (1979) *Curr. Top. Cell. Regul.* **15**, 17–107
- Weiss, G. B. (1974) *Annu. Rev. Pharmacol.* **14**, 343–354
- Willems, C., Rocmans, P. A. & Dumont, J. E. (1971) *FEBS Lett.* **14**, 323–325