Biochem. J. (1975) **150**, 305–314
Printed in Great Britain

Calcitonin-Sensitive Adenylate Cyclase in Rat Renal Tubular Membranes

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(*Received* 9 *December* 1974)

1. Renal tubular membranes from rat kidneys were prepared, and adenylate cyclase activity was measured under basal conditions, after stimulation by NaF or salmon calcitonin. Apparent K_m value of the enzyme for hormone-linked receptor was close to 1×10^{-8} M. 2. The system was sensitive to temperature and pH. pH was found to act both on affinity for salmon calcitonin-linked receptor and maximum stimulation, suggesting an effect of pH on hormone–receptor binding and on a subsequent step. 3. KCl was without effect whereas CoCl and $CaCl_2$ above 100 μM and $MnCl_2$ above 1 μM inhibited F^-- and salmon calcitonin-sensitive adenylate cyclase activities. The Ca^{2+} inhibition of the response reflected a fall in maximum stimulation and not a loss of affinity of salmon calcitonin-linked receptor for the enzyme. 4. The measurement of salmon calcitonin-sensitive adenylate cyclase activity as a function of ATP concentration showed that the hormone increases the maximum velocity of the adenylate cyclase. GTP, ITP and XTP at 200 μM did not modify basal, salmon calcitonin- and parathyroid hormone-sensitive adenylate cyclase activities. 5. Basal, salmon calcitonin- and F^--sensitive adenylate cyclase activities decreased at Mg^{2+} concentrations below 10 mM. High concentrations of Mg^{2+} (100 mM) led to an inhibition of the F^--stimulated enzyme. 6. Salmon calcitonin-linked receptor had a greater affinity for adenylate cyclase than human or porcine calcitonin-linked receptors. There was no additive effect of these three calcitonin peptides whereas parathyroid hormone added to salmon calcitonin increased adenylate cyclase activity, thus showing that both hormones bound to different membrane receptors. Human calcitonin fragments had no effect on adenylate cyclase activity. 7. Salmon calcitonin-stimulated adenylate cyclase activity decreased with the preincubation time. This was due to progressive degradation of the hormone and not to the rate of binding to membrane receptors.

It is well established that rat renal cortical homogenates contain an adenylate cyclase system that is stimulated by parathyroid hormone (Chase & Aurbach, 1968; Melson *et al.*, 1970; Forte, 1972; Beck *et al.*, 1972; Kurokawa & Massry, 1973) and calcitonin (Murad *et al.*, 1970; Melson *et al.*, 1970; Marx *et al.*, 1972*b*). Adenylate cyclase sensitivity to both hormones varies along the nephron. Parathyroid hormone acts mainly on adenylate cyclase present in glomeruli (Sraer *et al.*, 1974*b*; Imbert *et al.*, 1974) and outer cortex (Marx *et al.*, 1972*b*), whereas calcitonin is inactive in glomerular preparations (Sraer *et al.*, 1974*b*) but very effective in the inner part of the cortex (Marx *et al.*, 1972*b*). Previous studies (Marx *et al.*, 1972*a*; Sraer *et al.*, 1974*a*) have shown that ^{125}I-labelled salmon calcitonin binds specifically to renal membranes, which suggests that the binding initiates adenylate cyclase activation.

This study examines the principal characteristics of the calcitonin-sensitive adenylate cyclase present in renal tubular membranes. The effects of several parameters on basal, calcitonin- and F^--sensitive adenylate cyclase activities were compared. To separate modifications of receptor–hormone binding from changes involving only the enzyme, adenylate cyclase activation by salmon calcitonin was measured at various hormone concentrations and an apparent K_m value of the cyclase system for salmon calcitonin-linked receptor calculated from the dose–response curve. Modification of the apparent K_m value in the absence of change in basal and F^--sensitive activities was regarded as indicative of change in the binding of the hormone to its receptors (Bockaert *et al.*, 1972). Modification of maximum hormonal activity without any change in basal and F^--sensitive activities was interpreted as a more specific effect on receptor–cyclase coupling.

As parathyroid hormone and calcitonin have some renal effects in common, particularly diminution of phosphate and sodium tubular reabsorption, stimulation of adenylate cyclase by parathyroid hormone and calcitonin from different species was also compared, to establish possible hormonal interactions.

Table 1. *Evolution of* $(Na^+ + K^+)$*-activated ATPase and adenylate cyclase activities during enzyme preparations obtained from either cortex or medulla*

Values given are means ± S.E.M. Values in parentheses indicate the degree of purification. F^- and salmon calcitonin were added at 10mM and 10μM final concentration respectively.

	$(Na^+ + K^+)$-activated ATPase activity (μmol of P_i/h per mg of tissue)	Adenylate cyclase activity (pmol of cyclic AMP formed/10min per mg of tissue)	
		F^--stimulated	Salmon calcitonin-stimulated
Cortex			
Crude homogenate	2.79 ± 1.20	639.01 ± 227.33	179.54 ± 116.30
Purified membranes	7.77 ± 1.49 (2.78)	2996.52 ± 1084.10 (4.69)	865.21 ± 197.83 (4.82)
Medulla			
Crude homogenate	1.52 ± 0.25	466.06 ± 28.30	258.35 ± 10.25
Purified membranes	10.29 (6.77)	1607.98 ± 506.60 (3.45)	576.74 ± 121.97 (2.23)

Materials

The following materials were used: salmon calcitonin from Armour Montague (Paris, France) or Sandoz (Basel, Switzerland); human calcitonin and its fragments and 1,24-adrenocorticotrophin (Synacthen) from Ciba-Geigy (Basel, Switzerland); highly purified bovine parathyroid hormone and its synthetic 1–34 fragment from Wilson (Chicago, Ill., U.S.A.) and Beckman (Geneva, Switzerland) respectively; ATP (hydroxymethylaminomethane salt) from Sigma (St. Louis, Mo., U.S.A.); adenosine 3′:5′-cyclic monophosphate from Calbiochem (San Diego, Calif., U.S.A.); creatine kinase, phosphocreatine (disodium salt) from Boehringer (Mannheim, W. Germany); theophylline from Merck (Darmstadt, W. Germany); neutral aluminium oxide from M. Woelm (Eschwege, W. Germany); cyclic [^{3}H]-AMP (NH_4^+ salt, 27Ci/mmol) and [α-^{32}P]ATP (sodium salt, 960mCi/mmol) stored at −25°C in 50% (v/v) ethanol from The Radiochemical Centre (Amersham, Bucks., U.K.).

Methods

Enzyme preparation

Renal tubular membranes were prepared from male Charles River rats C.D. (150–200g) by the technique of Fitzpatrick *et al.* (1969), and used as the source of adenylate cyclase. Cortex and medulla were both used as initial material. The enzyme was utilized immediately after preparation or stored in liquid N_2 for 2 or 3 weeks. As shown in Table 1 the specific activities of the calcitonin- or F^--sensitive adenylate cyclase, and of the $(Na^+ + K^+)$-activated ATPase* were increased between 2.2 and 6.8 times, compared with the initial homogenates, in both cortical and medullary preparations.

Enzyme assays

Adenylate cyclase was measured by the technique of Bockaert *et al.* (1972), with minor modifications: ATP concentration was 1mM instead of 0.25mM; theophylline (9mM) and bovine serum albumin (3mg/ml) were added to the standard medium and incubations were carried out at 22°C unless otherwise stated; 1,24-adrenocorticotrophin (100μM) was also added to prevent degradation of salmon calcitonin (Marx *et al.*, 1973; Sraer & Ardaillou, 1974) or of other hormones in the presence of renal membranes after verification of its absence of effect on the adenylate cyclase activity. ATP and [α-^{32}P]ATP were added after a 20min preincubation. The reaction was stopped by adding 150μl of cold diluting solution composed of 50mM-Tris–HCl, pH7.4, 6.5mM-cyclic AMP, 3.3mM-ATP and $5\times10^{-3}$$\mu$Ci of cyclic [^{3}H]AMP.

Protein determinations were made by the method of Lowry *et al.* (1951). Adenylate cyclase activities were expressed as pmol of cyclic AMP formed/10min per mg of enzyme protein. $(Na^+ + K^+)$-activated ATPase was measured by the method of Post & Sen (1967) and the P_i released was measured by the method of Fiske & Subbarow (1925).

* Abbreviation: ATPase, adenosine triphosphatase.

Results

General properties of the adenylate cyclase system of renal cortical membranes

The amounts of cyclic AMP formed increased progressively with incubation time (0–20min). The addition of 10μM-salmon calcitonin or of 10mM-NaF to the incubation medium clearly increased cyclase activity. Basal and salmon calcitonin-sensitive adenylate cyclase curves were parallel from 5 to 20min whereas the F^--sensitive curve had a steeper slope (Fig. 1). Similarly, basal, salmon calcitonin- and F^--sensitive adenylate cyclase activities increased linearly with increasing enzyme concentrations over the range 0–172μg of tubular protein/tube (Fig. 2). The salmon calcitonin concentration yielding one-half of the maximum

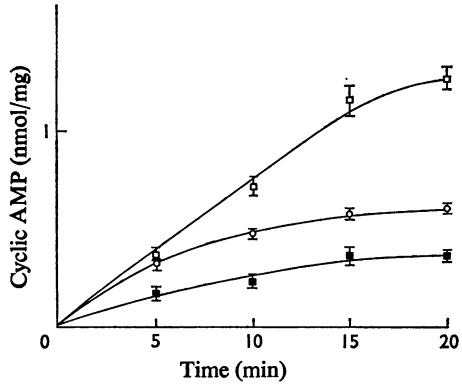

Fig. 1. *Accumulation of cyclic AMP as a function of time under basal conditions* (■) *or after stimulation by* F^- (10 mM; □) *or salmon calcitonin* (10 μM; ○)

Each point represents the mean and the vertical bars ± twice the S.E.M. for four values.

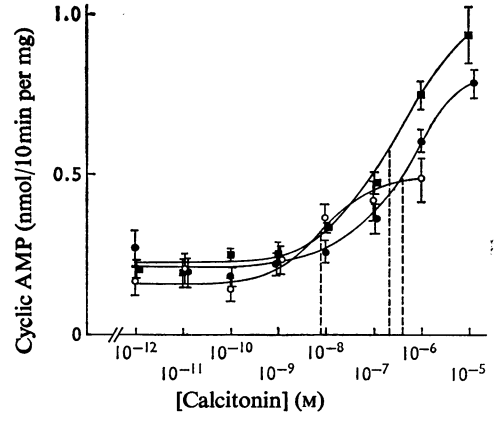

Fig. 3. *Activation of adenylate cyclase by salmon calcitonin* (○), *human calcitonin* (●) *and porcine calcitonin* (■)

Vertical lines indicate apparent K_m values (7.9×10^{-9}, 2.1×10^{-7} and 4.0×10^{-7} M for salmon, porcine and human calcitonin respectively). Each point is the mean and each vertical bar ± twice the S.E.M. for 10 values.

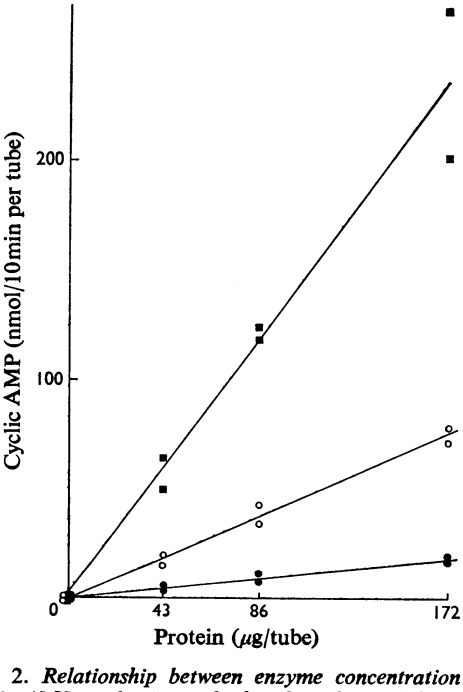

Fig. 2. *Relationship between enzyme concentration and cyclic AMP production under basal conditions* (●) *or after stimulation by* F^- (10 mM; ■) *or salmon calcitonin* (10 μM; ○)

The data are the results of one experiment with duplicate assays at each point.

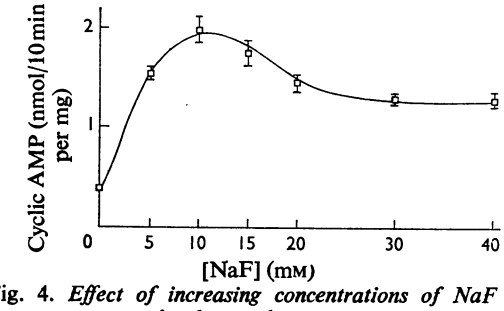

Fig. 4. *Effect of increasing concentrations of NaF on adenylate cyclase activity*

Each point is the mean and each vertical bar ± twice the S.E.M. for four values.

response (apparent K_m value) varied between 1.8 and 17 nM (Fig. 3). Maximum activation in the presence of F^- occurred at 10 mM (Fig. 4). Basal, F^-- and salmon calcitonin-sensitive adenylate cyclase activities varied with differing membrane preparations and the day of the study (Table 2).

Unless otherwise stated, all parameters were studied on cortical membrane preparations.

Effects of temperature and pH

Over a range of 4–46°C, the basal and salmon calcitonin-sensitive activities of the adenylate cyclase system were much less affected than the F^--sensitive activity (Fig. 5*a*). F^--stimulated adenylate cyclase activity increased from 4° to 37°C and fell pro-

Table 2. *Two-factor analysis of variance of adenylate cyclase activity performed on different days and with different membrane preparations*

Basal, F^- (10mM)- and salmon calcitonin (10μM)-sensitive activities were tested. Numbers in parentheses indicate the number of values. There is a significant variation due to the two factors tested. F values are given; **, $P<0.01$. Values of $m \pm t_{5\%} \times$S.E.M., which indicate the dispersion of the data for $P<0.05$, are given where m = mean and $t_{5\%} \times$S.E.M. = standard error of the mean multiplied by the t value read for 5% probability of non-significance and the degree of freedom corresponding to the number of values.

	Adenylate cyclase activity (pmol of cyclic AMP/10min per mg)		
	Basal	Salmon calcitonin-stimulated	F^--stimulated
$m \pm t_{5\%} \times$S.E.M.	260±28 (124)	641±51 (99)	2245±220 (98)
Variation between days	$F=4.97$**	$F=8.13$**	$F=22.57$**
Variation between membranes	$F=10.99$**	$F=8.98$**	$F=23.12$**

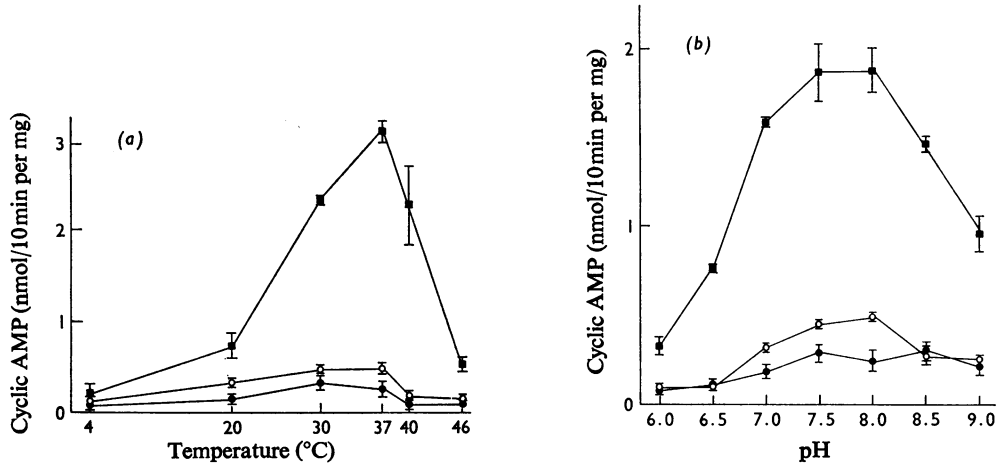

Fig. 5. *Effects of temperature (a) and pH (b) on basal* (●), *salmon calcitonin* (10μM; ○)- *and* F^- (10mM; ■)-*sensitive adenylate cyclase activity*

Each point is the mean and each vertical bar ± twice the S.E.M. for four values.

gressively between 37°C and 46°C, whereas basal and hormone-stimulated activities increased from 4° to 30°C, were not significantly modified between 30° and 37°C, then fell and reached very low values above 40°C.

As shown in Fig. 5(*b*), basal, salmon calcitonin- and F^--sensitive adenylate cyclase activities exhibited different pH-dependencies between pH6 and 9. Basal adenylate cyclase activity increased slightly from pH6 to 7.5 but remained unaffected between pH7.5 and 9. F^-- and salmon calcitonin-sensitive adenylate cyclase activities were maximum at pH7.5–8. Both activities fell at higher pH values. pH-dependence was greatest for F^--sensitive activity. Fig. 6 indicates that the loss of reactivity at pH6.5 compared with sensitivity at pH8 resulted both from a decreased apparent affinity of salmon calcitonin-linked receptor for the adenylate cyclase system and from a diminished maximum stimulation.

Effects of K^+, Co^+, Mn^{2+} *and* Ca^{2+} (*Table* 3)

KCl in concentrations between 1μM and 10mM had no effect on basal or F^-- or salmon calcitonin-sensitive adenylate cyclase activities. CoCl produced a decrease in F^-- and salmon calcitonin-sensitive adenylate cyclase activities at concentrations above 100μM, but had no effect at lower concentrations. Basal and salmon calcitonin-sensitive adenylate cyclase activities were not modified in the presence of increasing $MnCl_2$ concentrations over the range 1μM–10mM whereas F^--sensitive adenylate cyclase activity varied with a maximum at 1mM. Membranes

were prepared in a medium containing 1 mM-EDTA so that it was impossible to estimate the concentration of free Ca^{2+} in the incubation medium at the lower values of added Ca^{2+}. Basal and salmon calcitonin-sensitive activities did not change at $CaCl_2$ concentrations between 1 and 100 μM but fell at higher concentrations, whereas F^--sensitive activity diminished progressively over the entire range studied, 1 μM–10 mM. Experiments in which the dose–response curves of adenylate cyclase at different salmon calcitonin concentrations were measured for different Ca^{2+} concentrations showed that the Ca^{2+} inhibition of the response reflected a fall in maximum stimulation and not a lowered affinity of salmon calcitonin-linked receptor for the enzyme (Fig. 7).

Effects of nucleotides

Basal, F^-- and salmon calcitonin-sensitive adenylate cyclase activities were measured at various ATP concentrations. The velocity–substrate concentration curves (Fig. 8*a*) showed that stimulation by salmon calcitonin reflected increase in maximum reaction velocity and not a change in affinity of adenylate cyclase for ATP (K_m values 2×10^{-3} and 2.6×10^{-3} M for basal and salmon calcitonin-sensitive activity

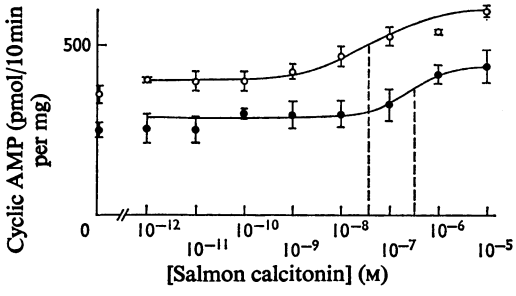

Fig. 6. *Effect of salmon calcitonin concentration on adenylate cyclase activity measured at pH* 6.5 (●) *and* 8.0 (○) *respectively*

Vertical dashed lines indicate apparent K_m values (3.7×10^{-8} and 3.2×10^{-7} M for pH 8 and 6.5 curve respectively). Each point is the mean and each vertical bar ± twice the S.E.M. for four values.

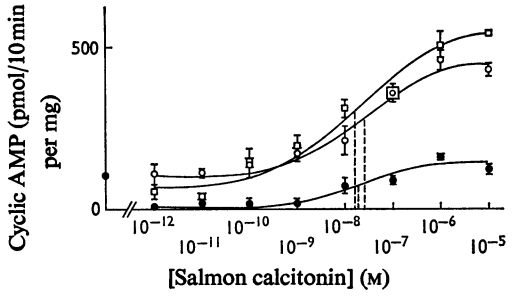

Fig. 7. *Effect of salmon calcitonin concentration on adenylate cyclase activity measured in the presence of* 1 mM-*EDTA* (□), 1 μM-$CaCl_2$ (○) *and* 1 mM-$CaCl_2$ (●) *respectively*

Each point is the mean and each vertical bar ± twice the S.E.M. for four values. Dashed vertical lines indicate the apparent K_m values (1.6×10^{-8}, 1.8×10^{-8} and 2.5×10^{-8} M for EDTA, 1 mM-$CaCl_2$ and 1 μM-$CaCl_2$ respectively).

Table 3. *Basal, salmon calcitonin- and F^--sensitive adenylate cyclase activities in the presence of increasing concentrations of different cations*

Values given are means of triplicates and expressed as pmol of cyclic AMP/10 min per mg.

Cation tested		Adenylate cyclase activity				
	Cation concentration ...	1 μM	10 μM	100 μM	1 mM	10 mM
K^+	Basal activity	162	158	171	222	171
	Salmon calcitonin-sensitive activity	637	699	656	653	667
	F^--sensitive activity	3198	3647	3581	3392	3745
Co^+	Basal activity	208	256	241	194	161
	Salmon calcitonin-sensitive activity	442	484	405	237	186
	F^--sensitive activity	2008	1935	2011	1274	516
Ca^{2+}	Basal activity	146	162	158	114	38
	Salmon calcitonin-sensitive activity	612	690	617	293	45
	F^--sensitive activity	3855	3448	3194	1772	282
Mg^{2+}	Basal activity	22	22	38	81	141
	Salmon calcitonin-sensitive activity	45	55	97	58	626
	F^--sensitive activity	32	322	326	574	3180
Mn^{2+}	Basal activity	222	279	255	247	297
	Salmon calcitonin-sensitive activity	222	267	163	353	91
	F^--sensitive activity	1219	1212	1223	1867	912

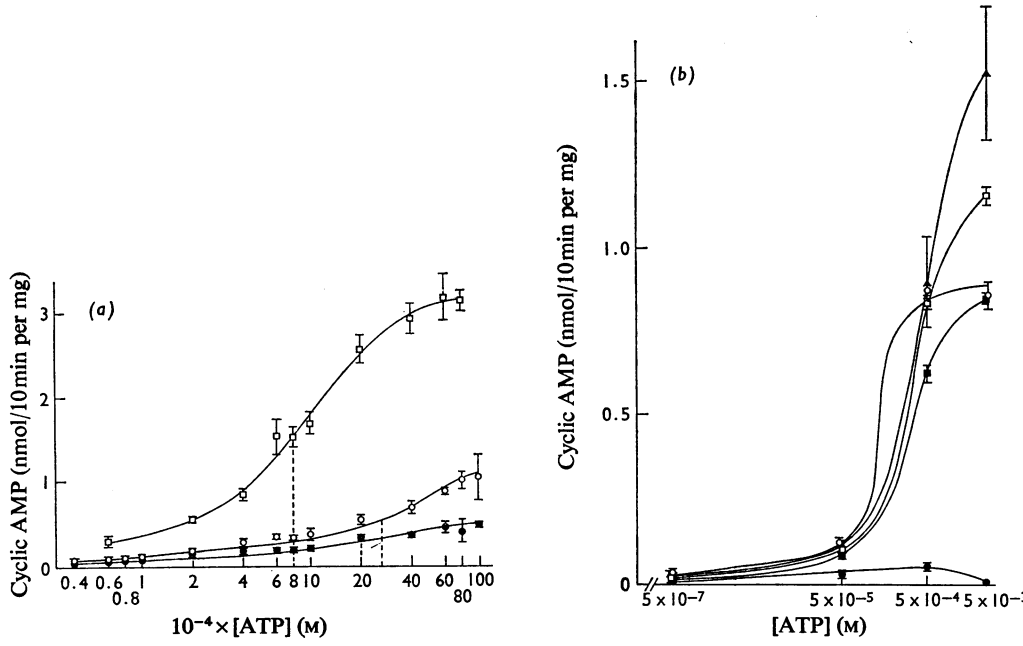

Fig. 8. *Effect of ATP concentration on basal* (●), F^- (10mM; □)- *and salmon calcitonin* (10μM; ○)*-sensitive adenylate cyclase activities* (*a*) *and on* F^- (10mM)*-sensitive adenylate cyclase activity at different* Mg^{2+} *concentrations* (1mM, ●; 5mM, ○; 10mM, □; 20mM, ▲; 100mM, ■) (*b*)

Each point is the mean and each vertical bar ± twice the S.E.M. for four values. Dashed vertical lines (*a*) indicate the apparent K_m values for ATP (8×10^{-4}, 2×10^{-3} and 2.6×10^{-3} M for F^--sensitive, basal and salmon calcitonin-sensitive activity respectively).

respectively). F^- stimulation, however, represented both increased affinity (K_m 8×10^{-4}M) and greater maximum reaction velocity. The calculated Hill coefficients were 0.88, 0.92 and 1.14 for the basal, F^-- and salmon calcitonin-sensitive activity respectively.

The possibility that the enzyme was a binding site for GTP, as described for hepatic glucagon-sensitive adenylate cyclase (Rodbell *et al.*, 1971), was tested. Basal adenylate cyclase activity was compared with activity in the presence of 0.2mM-GTP, -ITP and -XTP respectively, and salmon calcitonin- and parathyroid hormone-stimulated adenylate cyclase activities were compared with and without addition of the same nucleotides at an identical concentration. Student's *t* test showed the absence of effect of all these nucleotides.

Effects of Mg^{2+} *and ATP*

At constant ATP concentration of 1mM, salmon calcitonin- and F^--sensitive adenylate cyclase activities clearly decreased with Mg^{2+} concentrations below 10mM (Table 3), possibly owing to a low Mg^{2+}/ATP ratio producing an excess of free ATP. F^--sensitive adenylate cyclase activity was also measured as a function of ATP concentration in the presence of Mg^{2+} concentrations increasing from 1 to 100mM (Fig. 8*b*). At Mg^{2+} concentrations between 5 and 20mM, adenylate cyclase activation was observed, probably indicating that Mg^{2+}–ATP was the enzyme substrate (Birnbaumer *et al.*, 1969). A further increase in Mg^{2+} concentration (100mM) led to enzyme inhibition, possibly owing to formation of magnesium fluorophosphate. This complex is slightly dissociated and thus would lower the concentration of free Mg^{2+} (Bockaert *et al.*, 1972).

Effects of calcitonin from different species and of parathyroid hormone

Comparison of adenylate cyclase activation by salmon, human and porcine calcitonin showed maximum apparent enzyme affinity for salmon calcitonin (7.9nM), with values 25 and 50 times lower for porcine calcitonin (210nM) and human calcitonin (400nM) respectively (Fig. 3). When human calcitonin concentration was progressively increased in the presence of salmon calcitonin at concentrations 100 and 1nM respectively, adenylate cyclase activity remained constant provided stimulation due to

human calcitonin alone was lower than that of salmon calcitonin (Fig. 9). Thus the effects of these calcitonins were not additive. Similarly porcine calcitonin and salmon calcitonin, or porcine calcitonin and human calcitonin had no additive effects. Synthetic fragments of human calcitonin were also tested and did not significantly stimulate adenylate cyclase (Table 4). Purified bovine parathyroid hormone and its 1–34 synthetic fragment activated adenylate cyclase in a similar fashion (Fig. 10*a*). In both cases, maximum activity was seven to eight times basal activity and K_m values were in the range of 250–300 nM. Maximum activation produced by either of these parathyroid hormone peptides was higher than that observed with any form of calcitonin; but parathyroid hormone affinity, although in the

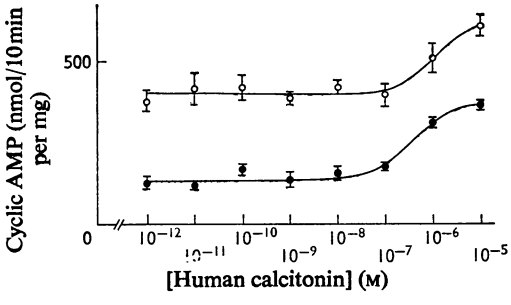

Fig. 9. *Effect of human calcitonin concentration on adenylate cyclase activity in the presence of either* 100 (○) *or* 1 (●) *nM-salmon calcitonin*

Table 4. *Adenylate cyclase activities in the presence of calcitonins from different species and of human calcitonin fragments at* 10 *μM final concentration*

Values given are means ± S.E.M. Numbers in parentheses indicate the number of values. Student's *t* test was performed to compare basal activity and activity in the presence of each peptide tested. N.S., Not significant.

	Adenylate cyclase activity (pmol of cyclic AMP/10min per mg)	Student's *t* test (peptide versus basal value)
Basal activity (no peptide present)	244 ± 17 (96)	
Peptide tested		
Salmon calcitonin	642 ± 36 (31)	$P<0.001$
Human calcitonin	607 ± 36 (24)	$P<0.001$
Porcine calcitonin	687 ± 57 (22)	$P<0.001$
11–28 Human calcitonin	113 ± 40 (2)	N.S.
11–32 Human calcitonin	140 ± 30 (3)	N.S.
17–28 Human calcitonin	162 ± 12 (3)	N.S.
17–32 Human calcitonin	134 ± 21 (2)	N.S.
21–28 Human calcitonin	229 ± 33 (6)	N.S.
29–32 Human calcitonin	194 ± 27 (3)	N.S.

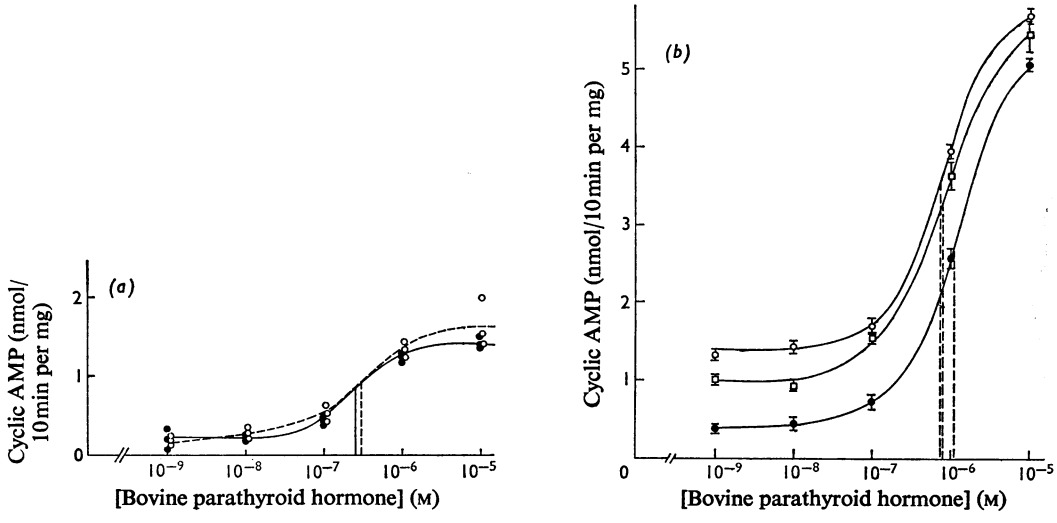

Fig. 10. *Effect on adenylate cyclase activity of purified bovine parathyroid hormone* (●) *and its* 1–34 *fragment* (○) (*a*) *in the presence* (1 *mM*, □, *and* 10 *μM*, ○) *and absence* (●) *of salmon calcitonin* (*b*)

The data are the results of two experiments with triplicate (*a*) and quadruplicate (*b*) assays. Vertical lines reaching the horizontal axis indicate apparent K_m values (2.5×10^{-7} and 3.0×10^{-7} M for purified bovine parathyroid hormone and its 1–34 fragment respectively, and 1.1×10^{-6}, 7.9×10^{-7} and 7.6×10^{-7} M for parathyroid hormone without and with 1 mM- or 10 *μM*-salmon calcitonin respectively, (*b*).

range of human and porcine calcitonin affinity, was clearly lower than salmon calcitonin affinity. Fig. 10(*b*) illustrates the additive effects of parathyroid hormone and calcitonins. When 1–34 bovine parathyroid hormone was added progressively in the presence of either 1 nM- or 10 μM-salmon calcitonin, the curves obtained remained horizontal only at 1–34 bovine parathyroid hormone concentrations which themselves had no effect. Maximum values were much higher than those observed in the presence of salmon calcitonin or 1–34 bovine parathyroid hormone alone. To test any potentiation between salmon calcitonin and parathyroid hormone, adenylate cyclase activity was measured in four experimental conditions: (1) presence of 1–34 bovine parathyroid hormone (10 μM); (2) presence of salmon calcitonin (10 μM); (3) presence of these two peptides at identical concentrations; (4) basal activity with no peptide added. Factorial analysis of variance did not reveal any significant difference between adenylate cyclase activity in the presence of 1–34 bovine parathyroid hormone plus salmon calcitonin and the sum of the activities obtained with each of these hormones separately. The effects of α-(phentolamine) and β-(propranolol) blocking agents at 10 μM were tested. Neither drug modified basal adenylate cyclase activity. Propranolol but not phentolamine significantly inhibited parathyroid hormone-sensitive activity whereas phentolamine but not propranolol significantly inhibited salmon calcitonin-sensitive activity (Table 5).

Salmon calcitonin-stimulated adenylate cyclase activity decreased with increasing preincubation time (incubation in the presence of salmon calcitonin without ATP), but basal activity was not significantly modified (Fig. 11*a*). Theoretically, this could represent either degradation of salmon calcitonin with time or diminution of adenylate cyclase activity with progressive occupancy of salmon calcitonin receptors. To resolve this problem, salmon calcitonin preincubated in the presence of renal membranes was either left with the same membranes during the incubation time or added at the same concentration to fresh membranes. No difference was observed between the two preparations for various preincubation times (Fig. 11*b*). Thus adenylate cyclase

Table 5. *Parathyroid hormone- and salmon calcitonin-stimulated adenylate cyclase activities in the absence or in the presence of propranolol or phenoxybenzamine*

Values given are means ± S.E.M. Numbers in parentheses indicate the number of values. Student's *t* test was performed between hormone-stimulated activity and the same in the presence of propranolol or phenoxybenzamine. N.S., Not significant.

Drug tested	Adenylate cyclase activity (pmol of cyclic AMP/10 min per mg)	Student's *t* test
Salmon calcitonin	642 ± 36 (31)	
1–34 Bovine parathyroid hormone	1372 ± 60 (16)	
Salmon calcitonin + propranolol	586 ± 54 (7)	N.S. versus salmon calcitonin
Salmon calcitonin + phenoxybenzamine	333 ± 13 (5)	$P < 0.01$ versus salmon calcitonin
Parathyroid hormone + propranolol	1063 ± 82 (7)	$P < 0.01$ versus parathyroid hormone
Parathyroid hormone + phenoxybenzamine	1256 ± 28 (5)	N.S. versus salmon calcitonin

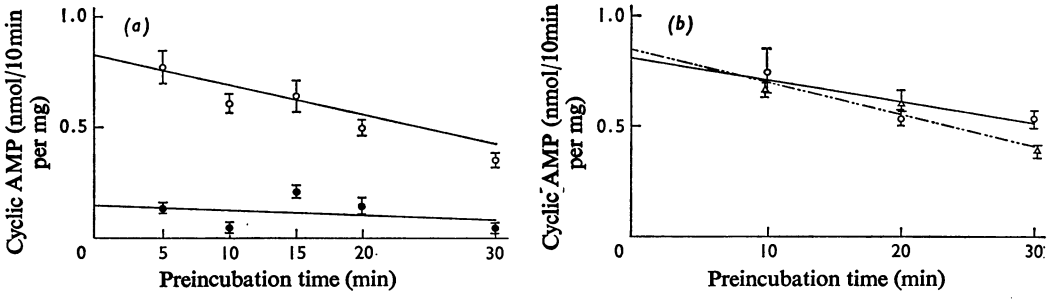

Fig. 11. *Effect of preincubation time on adenylate cyclase activity under basal conditions* (●) *or after stimulation by salmon calcitonin* (10 μM, ○)

Incubation was performed either with preincubated membranes and hormone (*a*) or with the preincubated hormone in the presence of either preincubated (○) or fresh membranes (△) (*b*). Each point represents the mean and each vertical bar ± twice the S.E.M. for four values.

activity did not depend on the degree of receptor occupancy, which was higher in the preparation containing preincubated membranes, but depended on the concentration of salmon calcitonin in the medium, which was identical in both preparations.

Discussion

These results confirm the presence of a calcitonin-sensitive adenylate cyclase system in renal cortical cells (Murad *et al.*, 1970; Melson *et al.*, 1970; Marx *et al.*, 1972*b*; Kurokawa *et al.*, 1974).

The action of F^- and parathyroid hormone on renal membrane adenylate cyclase was shown to be pH-dependent by Marcus & Aurbach (1971), who observed a maximum activity over the range pH 7–8. F^-- and salmon calcitonin-sensitive activities measured in our study were maximum over the same range. Moreover, inhibition that occurred with salmon calcitonin-stimulated adenylate cyclase at pH 6.5, compared with pH 8, reflected both a decrease in the apparent affinity of salmon calcitonin-linked receptor for the cyclase system and a modification in the maximum activity produced by this hormone. These results could be interpreted as an action of H^+ both on the binding of salmon calcitonin and on a subsequent step in the mediation of biological response.

As described by Marcus & Aurbach (1971), Ca^{2+} concentrations above 100μM inhibited basal and F^--sensitive adenylate cyclase. Salmon calcitonin-sensitive adenylate cyclase was also inhibited over the same range of Ca^{2+} concentration. Ca^{2+} does not act at the site of hormone–receptor binding, as the K_m value did not change with various Ca^{2+} concentrations (Fig. 7). Since maximum adenylate cyclase activity was inhibited, it is likely that Ca^{2+} affects a subsequent step. This hypothesis is compatible with the binding of hormone to receptor which must occur at physiological extracellular concentrations of free Ca^{2+} if these sites are extracellular. Ca^{2+} could act at the level of salmon calcitonin-linked receptor–cyclase coupling as suggested for oxytocin (Bockaert *et al.*, 1972) and adrenocorticotrophin (Lefkowitz *et al.*, 1970).

K^+ had no effect on basal, salmon calcitonin- and F^--sensitive adenylate cyclase in partial opposition to the results of Marcus & Aurbach (1971) who observed an activation of basal activity in male rat preparations but no effect on F^--stimulated activity in the presence of 50mM-KCl. CoCl inhibited salmon calcitonin- and F^--sensitive activities at concentrations above 0.1mM whereas $MnCl_2$ was inhibitory over the entire range of concentrations studied (1μM–10mM). Forte (1972) and Marcus & Aurbach (1971) found that Mn^{2+} and Co^+ could replace Mg^{2+} as cofactor but with a poorer specificity. As observed by Forte (1972), activation in the presence of F^- was maximum at 10mM.

The Mg^{2+} data confirm the conclusions of Marcus & Aurbach (1971) and Forte (1972) that Mg^{2+}–ATP plays a specific regulatory role in the enzymic system. Basal, salmon calcitonin- and F^--sensitive activities increase progressively with increasing concentrations of $MgCl_2$ from 1μM to 10mM; but, as shown in Fig. 10, Mg^{2+} concentration may be a limiting factor for the F^--sensitive adenylate cyclase activity at high ATP concentrations.

The apparent affinity of the enzyme for ATP was calculated from Michaelis–Menten curves under basal conditions and also in the presence of salmon calcitonin or F^-. Marcus & Aurbach (1971) were unable to obtain a plateau even at concentrations of ATP as high as 20mM, but Forte (1972) calculated a K_m value for ATP of 0.1mM for F^--sensitive adenylate cyclase, close to our observed value of 0.8mM.

Stimulation of tubular adenylate cyclase by porcine calcitonin (Melson *et al.*, 1970; Murad *et al.*, 1970; Kurokawa *et al.*, 1974) and salmon calcitonin (Marx *et al.*, 1972*b*; Sraer *et al.* 1974*a*) has been described. The apparent K_m (close to 10nM) in the present study is much higher than the physiological hormonal concentration and even higher than the apparent dissociation constant measured for binding of ^{125}I-labelled salmon calcitonin to renal membranes (Marx *et al.*, 1973; Sraer & Ardaillou, 1974; Sraer *et al.*, 1974*a*). Porcine, human and salmon calcitonin are active, the last-named having the greatest affinity, in agreement with previous studies (Marx *et al.*, 1973; Sraer & Ardaillou, 1974) showing greater inhibition of ^{125}I-labelled salmon calcitonin binding by salmon calcitonin than by either human or porcine calcitonin. Human calcitonin fragments have no effect on the adenylate cyclase system, which agrees with their total lack of biological activity *in vivo*. The effects of the three calcitonins are not additive, suggesting that they share identical receptor sites, whereas salmon calcitonin and parathyroid hormone effects are additive, indicating that these hormones have different receptors. Kurokawa *et al.* (1974) found no additive effect with parathyroid hormone and porcine calcitonin, but their results are difficult to reconcile with the great structural diversity of these two peptides. If parathyroid hormone and salmon calcitonin have additive effects, there is no potentiation as shown by the statistical analysis of the data.

Salmon calcitonin was kindly donated by Armour Montague (Paris, France) and Sandoz (Basel, Switzerland), and human calcitonin and its fragments by Ciba-Geigy (Basel, Switzerland). We are grateful to Professor Jard (Paris) for his advice and his interest in this work. We thank Dr. J. Whitworth for reviewing the English version and Miss M. Bizien for secretarial assistance.

References

Beck, N. P., De Robertis, F. R., Michelis, M. F., Fusco, R. D., Field, J. B. & Davis, B. B. (1972) *J. Clin. Invest.* **51**, 2352–2358

Birnbaumer, L., Pohl, S. L. & Rodbell, M. (1969) *J. Biol. Chem.* **244**, 3468–3476

Bockaert, J., Roy, C. & Jard, S. (1972) *J. Biol. Chem.* **247**, 7073–7081

Chase, L. R. & Aurbach, G. D. (1968) *Science* **159**, 545–547

Fiske, C. H. & Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–400

Fitzpatrick, D. F., Davenport, G. R., Forte, L. & Landon, E. J. (1969) *J. Biol. Chem.* **241**, 3561–3569

Forte, L. R. (1972) *Biochim. Biophys. Acta* **266**, 524–542

Imbert, M., Chabardes, D. & Morel, F. (1974) *Mol. Cell. Endocrinol.* **1**, 295–304

Kurokawa, K. & Massry, S. G. (1973) *Proc. Soc. Exp. Biol. Med.* **143**, 123–126

Kurokawa, K., Nagata, N., Sasaki, M. & Nakane, K. (1974) *Endocrinology* **94**, 1514–1518

Lefkowitz, R. J., Roth, J. & Pastan, I. (1970) *Nature (London)* **228**, 864–866

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Marcus, R. & Aurbach, G. D. (1971) *Biochim. Biophys. Acta* **242**, 410–421

Marx, S. J., Fedak, S. A. & Aurbach, G. D. (1972*a*) *J. Biol. Chem.* **247**, 6913–6918

Marx, S. J., Woodard, C. J. & Aurbach, G. D. (1972*b*) *Science* **178**, 999–1000

Marx, S. J., Woodward, C., Aurbach, G. D., Glossman, H. & Keutmann, H. T. (1973) *J. Biol. Chem.* **248**, 4797–4802

Melson, C. L., Chase, L. W. & Aurbach, G. D. (1970) *Endocrinology* **86**, 511–518

Murad, F., Brewer, H. B., Jr. & Vaughan, M. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **65**, 446–453

Post, R. L. & Sen, A. K. (1967) *Methods Enzymol.* **10**, 762–768

Rodbell, M., Birnbaumer, L., Pohl, S. L. & Krans, H. M. J. (1971) *J. Biol. Chem.* **246**, 1877–1882

Sraer, J. & Ardaillou, R. (1974) in *Endocrinology 1974: Proc. Int. Symp. 4th* pp. 170–176, Heinemann Medical Books, London

Sraer, J., Ardaillou, R. & Couette, S. (1974*a*) *Endocrinology* **96**, 632–637

Sraer, J., Ardaillou, R., Loreau, N. & Sraer, J. D. (1974*b*) *Mol. Cell. Endocrinol.* **1**, 285–294