Modulation of cyclic AMP action in the dog thyroid by its agonist and antagonist S_p - and R_p -adenosine 3',5'-monophosphorothioate

Christophe ERNEUX, Jacqueline VAN SANDE, Bernd JASTORFF and Jacques E. DUMONT

Institut de Recherche Interdisciplinaire (IRIBHN), Université Libre de Bruxelles, Hôpital Erasme, B-1070 Bruxelles, Belgium, and Fachbereich Biologie-Chemie, Universität Bremen, 2800 Bremen 33, Federal Republic of Germany

The diastereoisomeric forms of adenosine 3',5'-monophosphorothioate, S_p -cAMPS and R_p -cAMPS, have been shown to mimic and to inhibit activation of protein kinase type I and type II by cyclic AMP. In the present work, S_p -cAMPS mimicked thyrotropin (TSH) action on thyroid hormone secretion and protein iodination in dog thyroid slices, whereas R_p -cAMPS antagonized those effects. The phosphorothioates have been tested as inhibitors or activators of the three major phosphodiesterases: the Ca^{2+} /calmodulin-sensitive form, the cyclic GMP-stimulated form and the cyclic AMP-specific enzyme. At 100 μ M S_p -cAMPS inhibited the three enzyme activities. In contrast, R_p -cAMPS failed to stimulate activity of the three enzymes. From a comparison of the biological properties of S_p - and R_p -cAMPS and 3-isobutyl-1-methyl xanthine, it is suggested that one site of action of the phosphorothioates is on the cyclic AMP-dependent protein kinases, i.e. the effects of S_p -cAMPS and R_p -cAMPS observed in intact cell can be ascribed to the agonistic and antagonistic effects on the cyclic AMP-dependent protein kinases. However, partial inhibition of phosphodiesterase activities by the phosphorothioates cannot be excluded.

INTRODUCTION

Thyrotropin (TSH), by rapidly increasing the intracellular concentration of cyclic AMP, activates thyroid hormone synthesis and secretion (Dumont, 1971). Other positive effectors of adenylate cyclase, cholera toxin or forskolin, and cyclic AMP itself, reproduce this action in dog thyroid slices (Rodesch *et al.*, 1969; Van Sande *et al.*, 1979, 1982).

Two analogues of cyclic AMP have been synthesized in which one of the two exocyclic oxygens has been replaced by a sulphur atom, either in the axial position as S_p -cAMPS or in the equatorial position as R_p -cAMPS (Baraniak *et al.*, 1979). The S_p isomer binds to cyclic AMP-dependent protein kinase type I from rabbit skeletal muscle with approx. 10% of the binding affinity of cyclic AMP, but fully activates the protein kinase; in contrast, the R_p isomer does not activate the kinase at all, even with an occupancy of more than 90% (De Wit *et al.*, 1982, 1984). This R_p -cAMPS isomer antagonizes the activation by cyclic AMP of mammalian protein kinases type I and type II (De Wit *et al.*, 1984). In intact cells, e.g. isolated rat hepatocytes, S_p -cAMPS has been shown to stimulate glycogenolysis, and R_p -cAMPS to inhibit S_p -cAMPS- or glucagon-induced activation of glycogenolysis (Rothermel *et al.*, 1983, 1984).

Another possibility of interaction for S_p - and R_p -cAMPS is on the cyclic nucleotide phosphodiesterases. Mammalian phosphodiesterases consist of three prominent forms: a Ca^{2+} /calmodulin-sensitive enzyme, a cyclic GMP-stimulated form and a cyclic AMP-specific activity, the latter one sometimes controlled by hormones (e.g. by insulin) (Wells & Hardman, 1977). The inhibitory

potencies of S_p - and R_p -cAMPS on purified phosphodiesterases have not been studied. In the present report, we show in another well-characterized intact cell system, i.e. dog thyroid slices, that S_p -cAMPS reproduces the TSH-induced, cyclic AMP-mediated, stimulation of secretion and iodination, whereas R_p -cAMPS inhibits these effects.

MATERIALS AND METHODS

Materials

TSH (Thyropar) was obtained from Armour Pharmaceuticals (Phoenix, AZ, U.S.A.). Radioactive materials were provided by The Radiochemical Centre (Amersham). The phosphorothioates were prepared as in Baraniak *et al.* (1979) and purified by h.p.l.c. (Van Haastert, 1981). All of the other reagents were of the highest purity commercially available (Erneux *et al.*, 1980; Miot *et al.*, 1983).

Purification of enzymes and phosphodiesterase assay

The cyclic GMP-stimulated phosphodiesterase was purified to homogeneity from bovine adrenal tissue following the procedure described by Miot *et al.* (1985). The Ca^{2+} /calmodulin-sensitive phosphodiesterase was isolated from bovine brain as described earlier (Couchie *et al.*, 1983). The dog thyroid cyclic AMP-specific phosphodiesterase was obtained by DEAE-cellulose chromatography of a crude supernatant fraction (Miot *et al.*, 1983). The purified enzymes were stored at -80°C in the presence of 2 mg of bovine serum albumin/ml.

Assay of cyclic nucleotide phosphodiesterase activity

Abbreviations used: Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; TSH, thyrotropin; MIX, 3-isobutyl-1-methylxanthine; cAMPS, adenosine 3',5'-monophosphorothioate; dbcAMP, dibutyryl cyclic AMP; 8-Br-cAMP, 8-bromo cyclic AMP; I_{50} , concentration of analogue required to produce 50% inhibition of the hydrolysis of 1 μ M substrate (cyclic AMP).

was performed as described in Erneux *et al.* (1980). All the results are means of triplicate determinations. Protein concentration was measured according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Slice incubation and iodine metabolism

Thyroid slices were prepared with a Stadie Riggs microtome (Arthur Thomas, Philadelphia, PA, U.S.A.) from thyroids of dog pretreated with thyroid extract daily for 1–3 days (100 mg/10 kg; Thyranon, Organon, Oss, The Netherlands).

Within 30 min, they were incubated at 37 °C in Krebs–Ringer bicarbonate buffer (pH 7.4), saturated with O₂/CO₂ (19:1) and enriched with 8 mM-glucose and 0.5 g of bovine serum albumin/l. The slices were always preincubated for 60 min before the test incubation in fresh medium.

To measure thyroid hormone secretion, dogs were injected with 150 µCi of ¹³¹I on day 1 and received thyroid extract on day 2. The thyroid lobes were quickly removed on day 3, sliced and placed in the incubation medium (2 ml/vial) supplemented with NaClO₄ (1 mM) and methimazole (2 mM) to avoid iodide recirculation during the incubation. After 4 h incubation, the slices and 1 ml of medium were counted separately. The 1 ml of medium was then extracted with butanol as previously described (Unger *et al.*, 1979). Secretion was estimated by the ratio between the radioactivity extracted from the medium at the end of the 4 h incubation, and the total radioactivity present in the slices before the incubation (i.e. c.p.m. of ¹³¹I in medium and tissue at the end of the incubation). The results are expressed as a percentage of total radioactivity (Dumont *et al.*, 1971).

For the measurement of iodide incorporation into proteins, dog thyroid slices were incubated for 45 min in medium supplemented with KI (40 µM) and ¹³¹I (0.5 µCi/ml) and homogenized in a methimazole solution (2 mM). The proteins were precipitated with 5% (w/v) trichloroacetic acid (Rodesch *et al.*, 1969). Results were expressed as the ratio between trichloroacetic acid-precipitable radioactivity (protein-bound ¹³¹I) to the total radioactivity present in the slices from which the homogenate was made (percentage of total).

RESULTS

S_p-cAMPS mimicked TSH action on thyroid hormone secretion and protein iodination

Under appropriate conditions, TSH stimulates thyroid hormone secretion and protein iodination. Those effects are secondary to the activation of thyroid adenylate cyclase by TSH and to an increased intracellular cyclic AMP accumulation. In particular, dbcAMP, a cyclic AMP analogue that penetrates the cell and activates cyclic AMP-dependent protein kinase, reproduces those effects of TSH on thyroid metabolism (Rodesch *et al.*, 1969).

The stimulation by TSH (0.1–3 m-unit/ml) of thyroid hormone secretion and protein iodination was mimicked by 10–500 µM-S_p-cAMPS and was concentration-dependent (Figs. 1–3). The effect of S_p-cAMPS was comparable with that of other cyclic AMP analogues that stimulate protein kinase activity and have been used to validate the cyclic AMP model of hormonal action,

Fig. 1. Stimulation of hormone secretion in dog thyroid slices incubated in the presence of either S_p-cAMPS, 8BrcAMP, dbcAMP or TSH

After a preincubation of 1 h, the slices were transferred to fresh medium and incubation was carried out for 4 h in the presence of the stimulating agents (as indicated). The phosphodiesterase inhibitor Ro 20-1724 (0.1 mM) was also added to all vials at the beginning of the incubation. Secretion was measured as described in the Materials and methods section. Results are means of triplicates ± S.D. in one typical experiment; C, control.

8-BrcAMP or dbcAMP (shown for secretion in Fig. 1). Protein iodination was increased in the presence of the S_p isomer already at a concentration of 10 µM (Fig. 3a). The stimulatory effect of S_p-cAMPS was also observed in slices incubated in the presence of low levels of TSH (0.1–0.2 m-unit/ml). As shown in Fig. 2(b), the secretion estimated in the presence of 0.2 m-unit of TSH/ml was further enhanced (45%) by the mere addition of 50 µM-S_p-cAMPS. The stimulatory effect of S_p-cAMPS was stereoselective and specific for the S_p isomer. The R_p isomer (R_p-cAMPS) did not enhance secretion or iodination whether TSH was present or not in the incubation medium. In the experiment shown in Fig. 2(a), thyroid secretion had been measured in slices incubated in the presence of S_p-cAMPS or R_p-cAMPS both at 500 µM: only the S_p isomer stimulated secretion, mimicking TSH action. A similar conclusion is reached in the experiment shown in Fig. 2(b) in which TSH (0.2 m-unit/ml) was present and the phosphorothioates were at 50 µM: we observed that the secretion was selectively enhanced by the S_p isomer. At 10 and 100 µM, MIX, a phosphodiesterase inhibitor that did not affect protein kinase activity, did not stimulate thyroid secretion either (results not shown).

R_p-cAMPS antagonized the TSH-stimulated secretion and protein iodination

R_p-cAMPS specifically antagonized the TSH-induced increase in secretion and protein iodination. The effect started at 50 µM-R_p-cAMPS but was reproducibly observed at 500 µM of the R_p isomer. In the typical

Fig. 2. Effect of S_p - and R_p -cAMPS on basal and TSH-stimulated secretion

Secretion was measured after 4 h incubation. Analogues were added at the beginning of the incubation. In (a), TSH was absent. In (b), TSH was added where appropriate 30 min after the incubation was initiated. Results are means of triplicates $\pm$ s.d. in one typical experiment; C, control.

Fig. 3. Effect of S_p - and R_p -cAMPS on basal and TSH-stimulated protein iodination

After a preincubation of 45 min, the slices were transferred to fresh medium and incubation was carried out for 105 min. S_p -cAMPS or TSH was added 45 min before the end of the incubation. In (b), R_p -cAMPS was present from the beginning of the incubation. Iodination was measured as described in the Materials and methods section. Results are means of triplicates $\pm$ s.d. in one typical experiment; C, control.

Fig. 4. Comparison between the inhibitory potencies of S_p - and R_p -cAMPS on three isolated phosphodiesterases

Phosphodiesterase activity was measured at 5 μ M-cyclic AMP in the presence of excess calmodulin and 1 mM- CaCl_2 for the Ca^{2+} /calmodulin-sensitive phosphodiesterase (a), at 3 μ M-cyclic AMP in the presence of 3 μ M-cyclic GMP for the cyclic GMP-stimulated enzyme (b), and at 1 μ M-cyclic AMP for the cyclic AMP-specific phosphodiesterase (c). Results are means of triplicates $\pm$ s.d. Specific activity (amount of cyclic AMP hydrolysed/min per mg) is plotted on the ordinate; C, control (no analogue); PDE, phosphodiesterase.

experiment shown in Fig. 2(b), with 500 μ M- R_p -cAMPS, the secretion induced by 0.2 m-unit of TSH/ml was reduced by 40%. The effect was even more striking on protein iodination. As shown in Fig. 3(b), in the presence of 500 μ M- R_p -cAMPS, iodination was reduced by 60% and by 30% in thyroid slices stimulated by 0.5 and 10.0 m-unit of TSH/ml, respectively.

Inhibitory potencies of S_p - and R_p -cAMPS on three mammalian phosphodiesterases

We have compared the inhibitory potencies of S_p -cAMPS and R_p -cAMPS on three isolated and prominent phosphodiesterase activities found in multiple tissues as in dog thyroid (Miot *et al.*, 1983). S_p - and R_p -cAMPS did not enhance, and hardly inhibited, the activity of the Ca^{2+} /calmodulin-sensitive form and the cyclic GMP-stimulated enzyme with I_{50} values above 100 μ M (Fig. 4). MIX showed I_{50} values of 3 and approx. 20 μ M for the same type of enzymes, respectively (Miot *et al.*, 1984). Thus, compared with MIX, the phosphorothioate analogues of cyclic AMP were not very potent inhibitors. We came to the same conclusion with regard to the cyclic AMP-specific phosphodiesterase: although S_p -cAMPS was more effective than R_p -cAMPS to inhibit the hydrolysis of 1 μ M-cyclic AMP, MIX was nevertheless the most potent: I_{50} values were 100 μ M for the S_p analogue (Fig. 4) and 13 μ M for MIX (Miot *et al.*, 1984). The inhibition of phosphodiesterase activity by 500 μ M- R_p -cAMPS at 1 μ M-cyclic AMP was 57% for the cyclic AMP-specific phosphodiesterase. The R_p and S_p isomers of cAMPS did not stimulate cyclic AMP hydrolysis by the

cyclic GMP-stimulated phosphodiesterase (with 3 μ M-cyclic AMP as substrate in the absence of cyclic GMP, and up to 500 μ M of each analogue; results not shown).

DISCUSSION

In dog thyroid, most of the effects of TSH are mediated by an increase of intracellular cyclic AMP. In particular, exogenous added cyclic AMP or dbcAMP mimicked TSH action on secretion and iodination (Dumont, 1971; Rodesch *et al.*, 1969; Van Sande *et al.*, 1979, 1982). We show here the differential effects of S_p - and R_p -cAMPS both on secretion and iodination: S_p -cAMPS mimicked TSH action, whereas R_p -cAMPS was able to lower the TSH-induced stimulation of thyroid metabolism. Since the two analogues are diastereoisomers of adenosine 3',5'-phosphorothioate, their behaviour in intact cells can be classified as stereospecific. Previous reports on the interaction of those analogues with purified cyclic AMP-dependent protein kinase have also demonstrated a specific type of interaction: S_p -cAMPS activates both type I and type II protein kinases, with half-maximal activation constants in the micromolar range, and has been classified as a full agonist for those proteins (Van Haastert *et al.*, 1984). On the contrary, R_p -cAMPS is completely inactive in protein kinase activation (type I and type II), but inhibits the stimulatory effect of cyclic AMP in a competitive way with K_i values of 8 and 4 μ M for protein kinases type I and type II, respectively (De Wit *et al.*, 1984; Van Haastert *et al.*, 1984). The molecule has

been classified as competitive full antagonist of cyclic AMP for those proteins (Van Haastert *et al.*, 1984).

The stimulatory effect of S_p -cAMPS observed on iodination or secretion could result from two mechanisms: an activation of cyclic AMP-dependent protein kinase activities as suggested in hepatocytes (Rothermel *et al.*, 1984) or an inhibition of phosphodiesterase activity which would allow endogenous cyclic AMP to accumulate. The latter mechanism is unlikely to be involved: MIX, a more potent phosphodiesterase inhibitor than the S_p isomer, does not stimulate basal secretion at 10 or 100 μ M, i.e. at levels far above their I_{50} for calmodulin-dependent and cyclic GMP-activated phosphodiesterases. On the other hand, S_p -cAMPS at 100 μ M, i.e. below its I_{50} for these enzymes, enhanced secretion (up to 6-fold in Fig. 1).

On the other hand, the inhibitory effect of R_p -cAMPS could conceivably result from its known action as competitor of cyclic AMP for the kinases or from activation of phosphodiesterases. Since the R_p isomer did not activate the cyclic GMP-stimulated phosphodiesterase, nor the two other enzymes, the latter mechanism is unlikely. However, we cannot exclude partial inhibition of phosphodiesterase activity which would allow endogenous cyclic AMP to accumulate and partly compensate protein kinase inhibition. The latter mechanism could explain why the inhibition of thyroid hormone secretion or iodination is only partial. Similar results were obtained by Rothermel *et al.* (1984) who measured inhibition of glucagon-induced glucose production by R_p -cAMPS in isolated rat hepatocytes. The maximal inhibition of glucagon-induced glucose production by the R_p isomer at 500 μ M was 57%.

In conclusion, we have shown that S_p - and R_p -cAMPS respectively mimic, and inhibit, cyclic AMP action on secretion and iodination in dog thyroid slices. These analogues can therefore be used as tools in the identification of cyclic AMP-dependent mechanisms in this tissue.

This work was realized under contract of the Ministère de la Politique Scientifique (Actions Concertées, Belgium). One of us (B.J.) was supported by grant Ja 246-2,3 from the Deutsche Forschungsgemeinschaft and a grant from the Fonds der

Chemischen Industrie. We are grateful to Mrs. Colette Moreau and Mr. Claude Massart for technical assistance and to Mrs. Danièle Leemans for the preparation of the manuscript.

REFERENCES

- Baraniak, J., Kinas, R. W., Lesiak, K. & Stec, W. J. (1979) *J. Chem. Soc. Chem. Commun.* 940–941
- Couchie, D., Petridis, G., Jastorff, B. & Erneux, C. (1983) *Eur. J. Biochem.* **136**, 571–575
- DeWit, R. J. W., Hoppe, J., Stec, W. J., Baraniak, J. & Jastorff, B. (1982) *Eur. J. Biochem.* **122**, 95–99
- DeWit, R. J. W., Hekstra, D., Jastorff, B., Stec, W. J., Baraniak, J., Van Driel, R. & Van Haastert, P. M. J. (1984) *Eur. J. Biochem.* **142**, 255–260
- Dumont, J. E. (1971) *Vitam. Horm.* **29**, 288–412
- Dumont, J. E., Willems, C., Van Sande, J. & Neve, P. (1971) *Ann. N.Y. Acad. Sci.* **185**, 291–316
- Erneux, C., Couchie, D. & Dumont, J. E. (1980) *Eur. J. Biochem.* **104**, 297–304
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Miot, F., Dumont, J. E. & Erneux, C. (1983) *FEBS Lett.* **151**, 273–276
- Miot, F., Erneux, C., Wells, J. N. & Dumont, J. E. (1984) *Mol. Pharmacol.* **25**, 261–266
- Miot, F., Van Haastert, P. J. M. & Erneux, C. (1985) *Eur. J. Biochem.* **149**, 59–65
- Rodesch, F., Neve, P., Willems, C. & Dumont, J. E. (1969) *Eur. J. Biochem.* **8**, 26–32
- Rothermel, J. D., Jastorff, B. & Bothelho, L. H. P. (1984) *J. Biol. Chem.* **259**, 8151–8155
- Rothermel, J. D., Stec, W. J., Baraniak, J., Jastorff, B. & Botelho, L. H. P. (1983) *J. Biol. Chem.* **258**, 12125–12128
- Unger, J., Boeynaems, J. M., Van Herle, A., Van Sande, J., Rocmans, P. & Mockel, J. (1979) *Endocrinology (Baltimore)* **105**, 225–231
- Van Haastert, P. J. M. (1981) *J. Chromatogr.* **210**, 229–254
- Van Haastert, P. M. J., Van Driel, R., Jastorff, B., Baraniak, J., Stec, W. J. & DeWit, R. J. W. (1984) *J. Biol. Chem.* **259**, 10020–10024
- Van Sande, J., Pochet, R. & Dumont, J. E. (1979) *Biochim. Biophys. Acta* **585**, 282–292
- Van Sande, J., Cochaux, P. & Dumont, J. E. (1982) *FEBS Lett.* **150**, 137–141
- Wells, J. N. & Hardman, J. G. (1977) *Adv. Cyclic Nucleotide Res.* **8**, 119–143

Received 18 February 1985/13 September 1985; accepted 24 October 1985