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INHIBITION OF THE CYCLIC AMP–ADENYLATE CYCLASE SYSTEM AND OF SECRETION BY HIGH CONCENTRATIONS OF ADENOSINE IN THE DOG THYROID*

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Abstract—Adenosine inhibits cyclic AMP accumulation in stimulated slices and adenylate cyclase in acellular preparations of dog thyroid. The onset of this inhibition is rapid, requires relatively high adenosine concentrations ($\geqslant 10\ \mu M$) and occurs with all activators tested (TSH, PGE_1, forskolin and cholera toxin). The manganous ion, which uncouples receptor and cyclase, enhances the inhibition by adenosine. The effect of 2′,5′-dideoxyadenosine, the high concentration of adenosine needed, the Mn^{2+} effect and the lack of reversal by methylxanthines all suggest that this effect bears on the "P"-site, i.e. on the cyclase itself. Adenosine also inhibits thyroid secretion, which shows that its effect bears on the follicular cells. However the fact that cyclic AMP and DB cyclic AMP induced secretion are also reduced by adenosine suggests that adenosine also inhibits cyclic AMP action.

Adenosine, a putative neurotransmitter [1], regulates the function, metabolism and cyclic AMP§ content of numerous tissues [2–4]. It also modulates adenylate cyclase and its responsiveness to various hormones in acellular preparations [5–8]. It affects adenylate cyclase via two external receptors and one internal site, depending on the cell type. The external receptors are inhibitory (Ri) with a high affinity or stimulatory (Ra) with a low affinity. Agonists for these receptors show a stringent requirement for the unmodified ribose moiety of adenosine, while permitting substitution at various positions of the purine ring [9, 10]. The internal site (P-site) inhibits adenylate cyclase. It has a low affinity and requires an unaltered purine moiety while permitting substitution at various positions of the ribose. Methylxanthines are antagonists of the Ri and Ra receptors but have no effect on the P-site [11]. Divalent cations have little effect on Ra, inhibit Ri and increase P-site interaction with adenosine [12].

In the thyroid, few data on adenosine action have been reported. Wolff and coworkers have identified the presence of "P"-sites in beef, as well as mouse and human thyroid membranes and suggest that the "R"-sites are virtually absent in these preparations [13]. Adenosine inhibits protein kinase activity in beef thyroid [14]. However there is no indication that the cells involved are the follicular cells. Maayan *et al.* [15] observed an inhibition of mouse thyroid T_4 release *in vitro* at very high adenosine concentrations (5–10 mM) but did not investigate the mechanism of this effect.

Our aim was to investigate the effect of adenosine on the dog thyroid, one of the best defined and most studied systems [16, 17], from the first interaction of TSH with its receptor adenylate cyclase to its final consequence, thyroid secretion.

MATERIALS AND METHODS

Thyroid slices were prepared with a Stadie Riggs microtome (Arthur Thomas, Philadelphia, PA) from dogs pretreated with thyroid extract daily for 1–3 days [100 mg/10 kg, Thyranon (Organon Oss, The Netherlands)]. Within 30 min after the thyroid resection, the slices (30–60 mg wet weight) were incubated at 37° under an atmosphere of O_2–CO_2 (95:5, v/v) in 2 ml of Krebs–Ringer bicarbonate buffer (pH 7.4) enriched with 8 mM glucose and 0.5 g/l. bovine serum albumin. After a preincubation of 60 min, the slices were transferred to fresh medium for the test incubation.

For cyclic nucleotide assays, the slices were immediately dropped into 1 ml of boiling deionized water for 4 min, homogenized and centrifuged. The supernatant was lyophilized and resolubilized in water (10–20 μl of H_2O per milligram wet weight) and cyclic AMP measured by the method of Gilman [18, 19]. Thyroid secretion was measured as previously described [20]. Dogs were injected with 150 μCi of ^{131}I prior to the treatment with thyroid extract. The incubation medium was supplemented with $NaClO_4$ (1 mM) and methimazole (2 mM). Thyroid secretion was estimated by the ratio between

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§ Abbreviations: TSH, thyroid-stimulating hormone; cyclic AMP, adenosine 3′,5′-monophosphate; DB cyclic AMP, N^6-2′-*O*-dibutyryl adenosine 3′,5′-monophosphate; IBMX, isobutylmethylxanthine; PGE_1, prostaglandin E_1; $PGF_{1\alpha}$, prostaglandin $F_{1\alpha}$.

the butanol-extractable radioactivity in the medium at the end of the incubation and the total radioactivity of the slices before the incubation (% BEI).

For the measurement of adenylate cyclase activity, the thyroid slices were chopped and homogenized at 4° in a Dounce tissue grinder containing 50 mM Tris–HCl, pH 7.4, buffer with 250 mM sucrose, 1 mM EDTA and 1 mM EGTA. The suspension was centrifuged for 5 min at 500 *g*. The pellet was discarded. The pellet of a 20-min 10,000 *g* centrifugation was washed once and then resuspended in a 50 mM Tris–HCl, pH 7.4, buffer at a protein concentration of 0.5–1.5 mg/ml. The adenylate cyclase assay was performed as described by Salomon *et al.* [21]: 50 μl of the particulate preparation was added to 50 μl of incubation medium containing 50 mM Tris–HCl buffer (pH 7.4), 30 mM NaCl, 5 mM phosphocreatine (disodium salt), 10 U/ml creatine kinase, 5 mM $MgCl_2$, 0.1 mM ATP, 0.1 mM EGTA, 0.1 mM GTP, 0.8 mM IBMX, 250 mM sucrose, 0.04% bovine serum albumin and 1×10^6–2×10^6 cpm of [α-^{32}P]ATP. The medium was prewarmed for 5 min before the addition of enzyme. The incubation lasted 10 min at 30° in a shaker and was stopped by the addition of 900 μl of a solution of 5.5% TCA, 100 μM cyclic AMP, 500 μM ATP and [^{3}H]cyclic AMP (about 50,000 cpm per tube). After centrifugation, to remove the precipitated proteins, cyclic AMP was purified by a sequential chromatography on Dowex AG 50 W-X4 (200–400 mesh), followed by neutral alumina columns. Protein concentration was estimated as described by Lowry [22] using bovine serum albumin as standard. Under the conditions used, cyclic AMP generation was linear both with time up to 20 min, and with protein concentration up to 120 μg per tube.

In each experiment, the activity of the reagents and the reactivity of the slices were checked using the binding of ^{131}I to proteins as an index [23]. All the experiments have been performed at least 3 times with triplicate samples for each condition studied. Results are expressed as means ± S.E.M. of triplicate sets of slices in one typical experiment. TSH, as thytropar, was obtained from Armour Pharmaceutical Company (Chicago, IL); adenosine, atropine sulfate and ATP were purchased from Sigma Chemical Company (St. Louis, MO); phentolamine was provided by CIBA (Basel, Switzerland); adenosine deaminase (200 U/mg protein), cyclic AMP and GTP by Boehringer Pharmaceuticals (Mannheim, West Germany); and forskolin by Hoechst Pharmaceuticals (Bombay, India). Norepinephrine was obtained from Calbiochem (Lucerne, Switzerland), carbachol (carbamylcholine) from K and K (Plain View, New York). Cholera toxin (Dr R. A. Finkelstein's preparation) was provided by Schwarzmann (Division of Becton-Dickinson & Co (Orangeburg, New York) and PGE_1 and $PGF_{1\alpha}$ by the Upjohn Co. (Kalamazoo, MI). The phosphodiesterase inhibitor Ro 20-1724 was a gift from Hoffman–La Roche (Nutley, New Jersey), IBMX was purchased from Aldrich Chemical Co. (Milwaukee, WI) and 2′,5′-dideoxyadenosine from P-L Biochemicals (Milwaukee, WI). Dowex AG 50W-X4 was obtained from Bio-Rad Laboratories (Richmond, CA) and neutral alumina from Macherey Nagel & Co. (Düren, West Germany). Cyclic [^{3}H]AMP and [α-^{32}P]ATP were provided by the Radiochemical Centre (Amersham, U.K.). All of the other reagents were of the highest purity commercially available.

RESULTS

Adenosine inhibited dog thyroid secretion in TSH-stimulated slices at 0.1 mM and more so at 2 mM. In a representative experiment, the basal secretion of dog thyroid slices was increased from 1.36 ± 0.024 to 5.14 ± 0.16% by 0.5 mU/ml TSH in a 4-hr incubation. 0.1 and 1 mM adenosine reduced the stimulated level to 4.2 ± 0.24 and 2.06 ± 0.11% respectively. The same inhibitory effect of adenosine was observed when other stimulators such as DBcAMP and cAMP + 1 mM theophylline were used (Table 1). Inosine (1 mM) had no effect. The basal secretion was not modified.

A low concentration of adenosine (0.1 μM) had no effect on basal as well as TSH-induced cyclic AMP accumulation. A clear inhibition was observed in TSH-stimulated slices at 0.1, 1 and 10 mM (Fig. 1). Such an effect was obtained whatever the stimulator of adenylate cyclase. In one representative experiment 1 mM adenosine decreased the cyclic AMP levels from 1466 ± 64 to 430 ± 44 (pmoles of cyclic AMP per 100 mg of wet weight tissue ± S.E.M.) in TSH (1 mU/ml) stimulated slices, from 1093 ± 63 to 302 ± 19 with PGE_1 (14 μM), from 895 ± 112 to 260 ± 2 with cholera toxin (1 μg/ml) and from 938 ± 11 to 677 ± 33 with forskolin (12.5 μM). The basal value was 51 ± 4. In three separate experiments, the relative decrease induced by adenosine was smaller for forskolin than for the other agents.

The onset of the inhibitory action of adenosine was rapid (3 min) but of long duration (2 hr) (Fig. 2).

Table 1. Inhibitory effect of adenosine (1 mM) on thyroid secretion induced by various stimulators

	TSH (0.5 mU/ml)	cAMP (2 mM)	DBcAMP (0.5 mM)
—	2.07 ± 0.07	2.61 ± 0.07	3.22 ± 0.08
Adenosine (1 mM)	1.40 ± 0.03	1.84 ± 0.18	2.42 ± 0.11
Inosine (1 mM)	2.02 ± 0.17	—	—

The basal secretion was 0.78 ± 0.084.

The test incubation in the presence of the stimulators lasted 4 hr. Adenosine and inosine were added 15 min before the stimulators. Cyclic AMP was used in the presence of 1 mM theophylline to protect it from destruction.

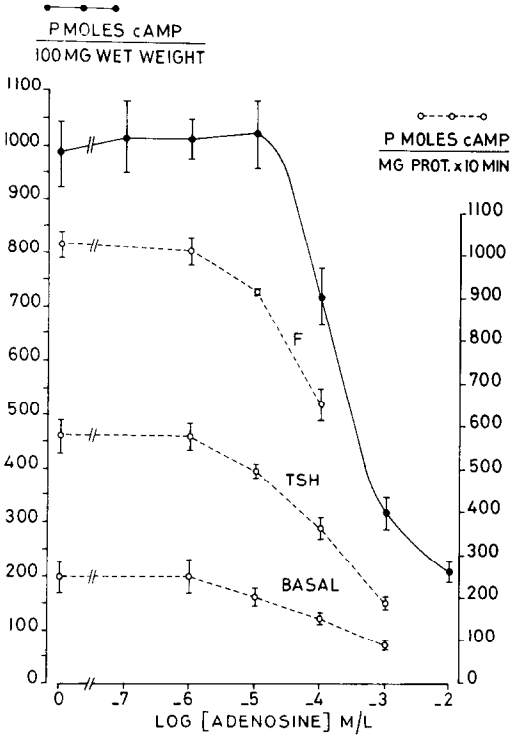

Fig. 1. Effect of increasing adenosine concentrations on cyclic AMP levels in TSH (1 mU/ml) stimulated dog thyroid slices and adenylate cyclase activity in membrane preparations. For cyclic AMP: the test incubation in the presence of adenosine, and IBMX (0.1 mM) as phosphodiesterase inhibitor, lasted 45 min. TSH was added 15 min after adenosine, for the last 30 min of the incubation. Basal value was 28 ± 1.1. Results are expressed as pmoles of cyclic AMP per 100 mg of wet weight tissue ± S.E.M. of triplicates in one typical experiment. For adenylate cyclase activity: the reaction was started with 30 μg of membrane proteins and was run for 10 min at 30°. Additions were: fluoride (F) (10 mM) and TSH (10 mU/ml). ATP, GTP and NaCl in the incubation mixture were respectively 0.1, 0.1 and 30 mM.

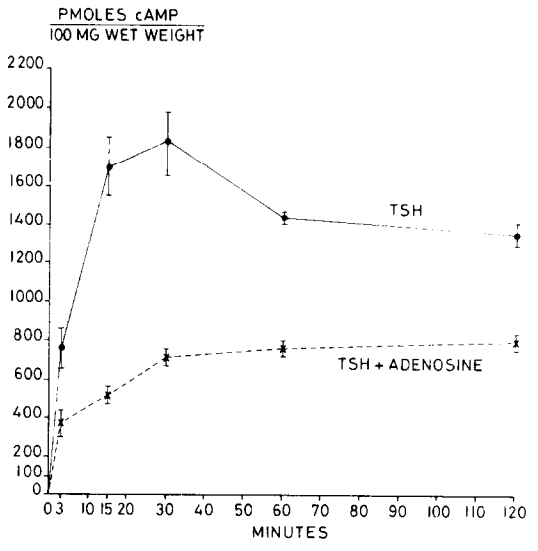

Fig. 2. Kinetics of the adenosine inhibitory effect on cyclic AMP accumulation in dog thyroid slices. The test incubation in the presence of adenosine (1 mM), TSH (1 mU/ml), and Ro 20-1724 (0.1 mM) as phosphodiesterase inhibitor, lasted from 3 to 120 min. For the shortest incubation times (3 min) Ro 20-1724 was already added 10 min before the end of the preincubation. Results are expressed in pmoles of cyclic AMP per 100 mg of wet weight tissue ± S.E.M. of triplicates of one representative experiment. The basal value was 68 ± 5.

Inhibition of acetylcholine and norepinephrine negative action [24–27] by the muscarinic and α-adrenergic receptor blocking agents, atropine (1 μM) and phentolamine (50 μM) respectively, had no effect on the action of adenosine (1 mM) on cyclic AMP accumulation in TSH-stimulated slices (data not shown). The inhibitory effect of carbamylcholine on cyclic AMP accumulation in TSH-stimulated slices is suppressed by methylxanthines, or in a Ca^{2+}-free incubation medium [25, 28]. The inhibitory effect of adenosine remained identical in the presence of IBMX (0.1 mM) or in a Ca^{2+}-depleted medium (data not shown). Carbamylcholine [24, 25], iodide [19], norepinephrine [26, 27] and $PGF_{1\alpha}$ [29] decrease cyclic AMP accumulation in dog thyroid stimulated slices. Adenosine deaminase (3 U/ml), which prevented the effect of 1 mM adenosine in our conditions, did not relieve the action of any of these inhibitors (data not shown).

Adenosine inhibited dog thyroid membrane adenylate cyclase activity with a threshold concentration at 10 μM (Fig. 1). Mn^{2+} activates adenylate cyclase and markedly enhances its inhibition by adenosine analogues at the P-site in beef thyroid membranes [13]. As for catecholamines in rat reticulocytes [30], in dog thyroid membrane preparations, the replacement of Mg^{2+} (5 mM) by Mn^{2+}

Table 2. Effect of the adenosine analog 2′,5′-dideoxyadenosine on thyroid adenylate cyclase activity

		Adenosine (100 μM)	2′,5′-Dideoxyadenosine (10 μM)	2′,5′-Dideoxyadenosine (100 μM)
—	148 ± 11	91 ± 5.7	71 ± 6.3	33 ± 0.6
TSH (10 mU/ml)	365 ± 5.2	189 ± 2.9	136 ± 1.1	63 ± 0.6
F^- (10 mM)	848 ± 15.6	551 ± 17	412 ± 8.6	164 ± 2.3

Incubation medium was as in Materials and Methods with 0.1 mM ATP and 30 mM NaCl. The reaction was started with 63 μg of membrane protein and was run for 10 min at 30°. Results are expressed as pmoles of cyclic AMP per milligram of protein per 10 min.
F^-: Fluoride.

(5 mM) in the incubation mixture enhanced the basal and fluoride-stimulated activity but suppressed the TSH and PGE_1 receptor mediated cyclase activation. In a representative experiment, basal and fluoride-stimulated activities were reduced by 0.1 mM adenosine from 319 ± 25 to 210 ± 7 and 1700 ± 88 to 1050 ± 10 pmoles per 10 min per milligram of protein in the presence of Mg^{2+} (5 mM), and from 575 ± 68 to 219 ± 8 and 2810 ± 63 to 775 ± 16 respectively in the presence of Mn^{2+} (5 mM).

2′5′-Dideoxyadenosine, the most active adenosine analog inhibitor at the P-site of adenylate cyclase [13], markedly inhibited the basal, TSH- and fluoride-stimulated adenylate cyclase activity (Table 2). This excludes an artefactual decrease in [^{32}P]cyclic AMP formation due to dilution by adenosine of the [^{32}P]ATP precursor. This analog was also more potent than adenosine itself on cyclic AMP accumulation in intact cells: 0.2 mM adenosine decreased cyclic AMP levels in TSH (1 mU/ml) stimulated slices from 1141 ± 152 to 473 ± 12 and 0.2 mM 2′5′-dideoxyadenosine reduced it to 298 ± 11 pmoles of cyclic AMP per 100 mg of wet weight tissue under the same conditions. The basal value was 148 ± 20.

DISCUSSION

Adenosine inhibited cyclic AMP accumulation in stimulated slices and adenylate cyclase in acellular preparations of dog thyroid. The fact that TSH-stimulated thyroid secretion was also reduced by the nucleoside suggests that the effect bears on the follicular cells. A similar effect of higher concentrations (5 mM) of adenosine on mouse thyroid has been observed [30]. However, these results and the observed inhibition of cyclase activity in beef thyroid membranes [13] are difficult to reconcile with the reported absence of an effect of high concentrations of adenosine (1–5 mM) on the cyclic AMP level in TSH-stimulated beef thyroid [14]. Perhaps a slower kinetics of adenosine penetration and action in beef slices did not allow the expression of an adenosine effect.

The effect of adenosine on dog thyroid adenylate cyclase is characterized by the following properties: obtained at relatively high adenosine concentrations; insensitive to methylxanthines; not selective for a particular stimulus of cyclase (TSH, fluoride and forskolin); reproduced by 2′5′-dideoxyadenosine, a P-site specific inhibitor; observed in the presence of Mn^{2+}, i.e. in receptor-uncoupled cyclase. The four former characteristics of adenylate cyclase inhibition by adenosine are similar to those of cyclic AMP depression in intact cells and suggest that the inhibition of cyclase by adenosine may account for its effect on cyclic AMP levels in thyroid slices. This inhibition takes place whether the stimulatory agent acts at the level of the receptor (TSH), of the transducing protein G/F (fluoride) or of the cyclase itself (forskolin). It operates on receptor-uncoupled cyclase. This suggests a point of action beyond the TSH receptor interaction step at the P-type site [12]. There is no indication of the presence of R-type sites. These data are in agreement with findings in beef thyroid membranes [13].

The inhibition of cyclic AMP accumulation by adenosine is independent of extracellular Ca^{2+} and insensitive to methylxanthines, atropine and phentolamine. The former characteristics exclude a mediation of the effect by acetylcholine, the latter a role of α-adrenergic receptors. On the other hand, adenosine deaminase does not antagonize the effects of acetylcholine, iodide, epinephrine and $PGF_{1\alpha}$, which suggests that adenosine does not mediate the action of these agents.

Previous *in vitro* studies have shown that adenosine inhibits protein kinases in some tissues including thyroid [14, 31–35]. The facts that cyclic AMP and DB cyclic AMP induced secretion were also reduced by adenosine suggest that adenosine also acts at this level in the sequence of cyclic AMP action. The high concentrations required to express these inhibitions of cyclic AMP formation and action are obviously in the pharmacological range.

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