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NEGATIVE REGULATION OF CYCLIC-AMP LEVELS BY CARBAMYLCHOLINE IN DOG THYROID IS NOT MEDIATED BY CYCLIC-GMP

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Abstract—Carbamylcholine, through calcium, enhances cyclic-GMP accumulation and depresses cyclic-AMP accumulation in TSH stimulated dog thyroid. The results presented show that compounds which can be transformed to nitric oxide increase cyclic-GMP accumulation in the dog thyroid. These compounds do not require extracellular calcium for their action. In thyroid stimulated by TSH, these compounds do not depress AMP accumulation. Cyclic-GMP is not the main intracellular signal involved in the negative regulation of cyclic-AMP levels in dog thyroid.

In dog thyroid, norepinephrine through an α_2 receptor [1] and carbamylcholine or acetylcholine through a muscarinic receptor [2, 3], both inhibit cyclic-AMP accumulation. The carbamylcholine effect is relieved by calcium depletion and by one phosphodiesterase inhibitor, isobutylmethylxanthine but not by another RO 20-1724 [4]. It has been related to an activation of cyclic-AMP catabolism [4]. On the contrary, the norepinephrine effect results from a classical direct inhibition of adenylate cyclase by the α receptor [1, 5]. Carbamylcholine enhances both calcium influx and cyclic-GMP accumulation in dog thyroid [3]. Its enhancement of cyclic-AMP disposal could therefore result from the activation of calmodulin dependent phosphodiesterase by calcium or of the cyclic-GMP activated phosphodiesterase by cyclic-GMP [6, 7]. In this work we show that treatment of dog thyroid with compounds which can be transformed to nitric oxide and activate guanylate cyclase, increases cyclic-GMP but does not depress cyclic-AMP accumulation in the cells. This shows that cyclic-GMP and cyclic-GMP activated phosphodiesterase are not the mediators involved in the negative regulation of cyclic-AMP accumulation in the thyroid.

MATERIALS AND METHODS

Thyroid slices from dogs pretreated with thyroid extract for 1 day (100 mg/10 kg, Thyranon, Organon Oss, The Netherlands), were prepared with a Stadie Riggs microtome (Arthur Thomas, Philadelphia, PA). Within 30 min after the thyroid resection, the slices (30–60 mg wet wt) were incubated at 37° under an atmosphere of O_2/CO_2 (95:5, v/v) in 2 ml Krebs–Ringer bicarbonate buffer (pH 7.4) enriched with 8 mM glucose. This medium contained $CaCl_2$ 1.45 mM. However, in some cases, a medium without added calcium (αCa^{2+}) and supplemented with EGTA (100 μM or 1 mM) was used. The experimental protocol involved a preincubation of 60 min to achieve steady-state of the cells *in vitro*. The slices were then transferred to fresh medium supplemented with the chemical required for the measurement of the metabolic variable. To inhibit cyclic-AMP and cyclic-GMP hydrolysis after the incubation, 1 mM caffeine was added 10 min before its end [8]. For cyclic-GMP and cyclic-AMP assays, the slices were immediately dropped into 1 ml of boiling deionized water for 5 min, homogenized and centrifuged. The supernatant was lyophilized and resolubilized in water (10 μl H_2O per mg wet weight). Cyclic-GMP concentration was measured by the slightly modified radioimmunoassay method of Cailla [9]. Succinylation was performed after dilution of the samples and separation of bound from free cyclic-GMP was carried out by precipitation of the bound nucleotide with isopropanol 1.1 ml for 0.3 ml medium (Merck, Schuchardt, FRG). This method allows detection of 2 fmole cyclic-GMP. The antibodies were provided by the Centre d'Immunologie of Luminy, Marseille, France. The tracer (iodinated 2'-0-succinyl-cyclic-GMP-tyrosine methyl ester) was prepared and purified in our laboratory. The inhibition curve gave the specificity of the antiserum anti-cyclic GMP: the percentage of cross reaction estimated by comparison of the concentration yielding a 50% binding inhibition was 0.002% for succinyl-cyclic-AMP. The cyclic-AMP was measured by the method of Gilman [10]. The results are expressed as mean ± S.D.M. of triplicate sets of slices in one typical experiment. All experiments have been carried out at least three times. TSH as thyrotropar was obtained from the Armour Pharmaceutical Company (Chicago, USA). Carbachol was obtained from K & K (Plain View, NY), sodium nitroprusside, from Merck (Art. 6541) and sodium azide from U.C.B. (Belgium). The phosphodiesterase inhibitor Ro 20-1724 was a gift

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Abbreviations: cyclic-AMP, adenosine 3',5'-monophosphate; cyclic-GMP, guanosine 3',5'-monophosphate; IBMX, 1-methyl-3-isobutylxanthine; EGTA, ethyleneglycol-bis-(β-amino-ethyl ether) *N,N'*-tetraacetic acid; TSH, thyroid-stimulating hormone.

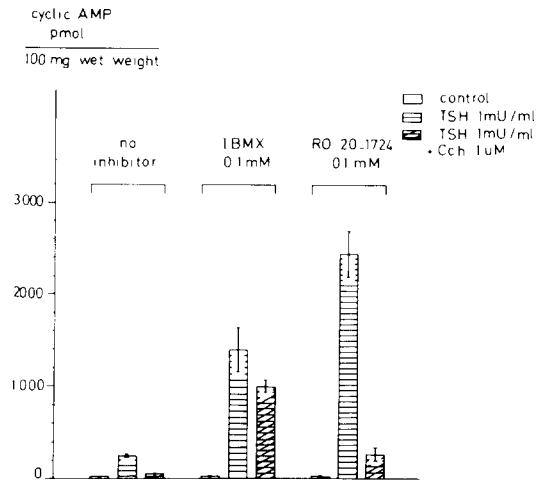


Fig. 1. The inhibition by carbamylcholine of the cyclic-AMP accumulation in TSH stimulated slices is relieved by IBMX but not by Ro 20-1724.

from Hoffman–La Roche (Nutley, New Jersey, USA), isobutylmethylxanthine (IBMX) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Cyclic-AMP, cyclic-GMP, 2′-0-succinyl-cyclic-GMP-tyrosine methyl ester, were obtained from Boehringer Pharma (Mannheim, FRG), cyclic [^{3}H] AMP from Amersham International (UK).

RESULTS

Carbamylcholine 10 μM stimulated cyclic-GMP accumulation and inhibited cyclic-AMP accumulation in dog thyroid slices stimulated by TSH. The latter effect was relieved by isobutylmethylxanthine 0.1 mM but not by Ro 20-1724 0.1 mM (Fig. 1). The negative control of carbamylcholine is thus not impaired in the presence of Ro 20-1724 which is a rather specific inhibitor of the cyclic-AMP high affinity phosphodiesterase [7, 11]. Experiments on the mechanism of the carbamylcholine effect can therefore be performed in the presence of this drug. As previously shown, carbamylcholine enhanced cyclic-GMP accumulation in the slices (Fig. 2). In seven experiments, TSH did not modify cyclic-GMP concentration in dog thyroid slices at concentrations from 1 to 10 mU/ml, after 2 to 240 min.

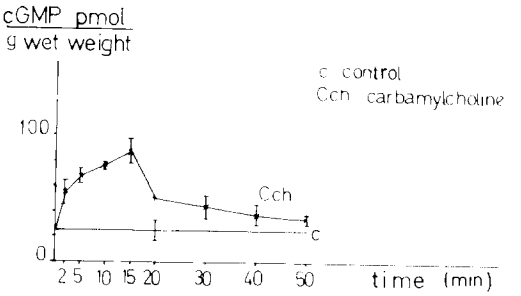


Fig. 2. Kinetics of accumulation of cyclic-GMP in presence of carbamylcholine (1 μM) from 2 to 50 min.

Several authors have shown that sodium azide, hydroxylamine, sodium nitroprusside, nitroglycerin, nitric oxide and nitrosoamines increase cyclic-GMP accumulation in various tissues [12–21]. These agents do not require extracellular calcium [15, 18, 22]. This cyclic-GMP accumulation would depend on oxidative mechanisms but not on calcium.

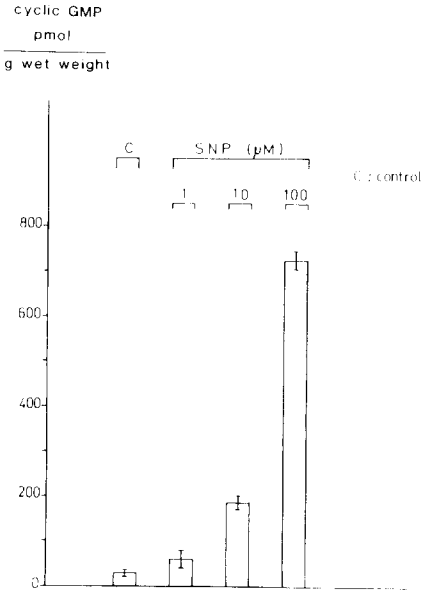


Fig. 3. Effect of increasing concentrations of sodium nitroprusside (SNP) (1–100 μM) on cyclic-GMP accumulation in slices preincubated 1 hr and incubated 2 min with sodium nitroprusside in a medium without added calcium and with 100 μM EGTA.

Table 1. Kinetics of action of sodium azide (NaN_3) (1 mM), hydroxylamine (NH_2OH) (1 mM) and sodium nitroprusside (SNP) (1 mM) on cyclic-GMP accumulation in slices incubated in a medium containing 1.45 mM calcium

Incubation time (min)	0	NaN_3 (1 mM)	NH_2OH (1 mM)	SNP (1 mM)
2	37 ± 7	—	—	2120 ± 237
3	—	93 ± 18	—	—
5	—	—	1314 ± 305	1520 ± 221
10	27 ± 1	118 ± 16	1054 ± 143	1518 ± 465

The results are expressed in pmoles of cyclic-GMP per g wet wt (±S.E.M.).

Table 2. Effect of hydroxylamine (NH_2OH) (1 mM) and sodium nitroprusside (SNP) (1 mM) on cyclic-GMP accumulation in slices incubated in a medium containing various concentrations of calcium

NH_2OH (1 mM)	SNP (1 mM)	Extracellular calcium	0
353 ± 100	881 ± 135	1.45 mM*	24 ± 1
326 ± 60	1210 ± 203	αCa^{2+}†	18 ± 1
383 ± 63	1238 ± 173	αCa^{2+} + EGTA 1 mM‡	8 ± 4

The slices were preincubated 1 hr in a medium: *containing 1.45 mM calcium; †without added calcium (αCa^{2+}); ‡without added calcium and with 1 mM EGTA and incubated 5 min in the same medium with the compound to be tested. The results are expressed in pmoles of cyclic-GMP per g wet wt (±S.E.M).

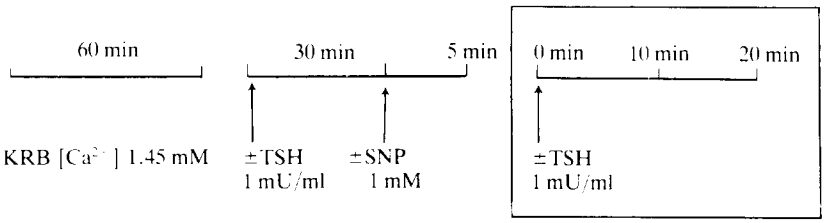
In dog thyroid slices, sodium azide (NaN_3), hydroxylamine (NH_2OH) and sodium nitroprusside (SNP), which can be transformed to nitric oxide [13, 14, 21, 23, 24], increased cyclic-GMP accumulation in a medium containing 1.45 mM calcium (Table 1). After 2 min of incubation, sodium nitroprusside greatly increased cyclic-GMP level. This effect was concentration-related (Fig. 3). It was reversible: in one typical experiment, control cyclic-GMP concentration of 23 ± 5 pmoles/g wet wt, increased to 197 after 2 min in the presence of sodium nitroprusside 100 μM; it decreased to 51 ± 24, 26 ± 6 and 29 ± 5 pmoles/g wet wt after 8, 28 and 48 min washing, respectively.

The increase in cyclic-GMP accumulation by hydroxylamine and sodium nitroprusside remained the same if the slices were incubated in a medium containing 1.45 mM calcium, no added calcium or depleted in calcium (in the presence of EGTA) (Table 2). As previously shown [3], cyclic-GMP levels in control slices decreased with extracellular calcium. These data demonstrate the existence, in dog thyroid, of the mechanism of positive regulation of cyclic GMP accumulation dependent on oxidative mechanisms.

In slices stimulated 35 min with TSH 1 mU/ml, treated 5 min with sodium nitroprusside, then transferred in a fresh medium with TSH 1 mU/ml, cyclic-AMP was not affected by sodium nitroprusside (Table 3). Sodium azide, hydroxylamine and sodium nitroprusside did not modify cyclic-AMP concentration in slices incubated 2, 3, 5 or 10 min in the presence of these agents (20–27 pmoles cyclic AMP per 100 mg wet wt) (data not shown).

As xanthines relieve the inhibition by carbamylcholine of the TSH induced cyclic-AMP accumulation, we have compared the effect of sodium nitroprusside induced cyclic-GMP accumulation on the TSH induced cyclic-AMP accumulation in the presence of these two phosphodiesterase inhibitors. Table 4 shows that in the presence of either IBMX or Ro 20-1724 and sodium nitroprusside, cyclic-GMP concentration strongly increased, but cyclic-AMP accumulation did not decrease. On the other hand,

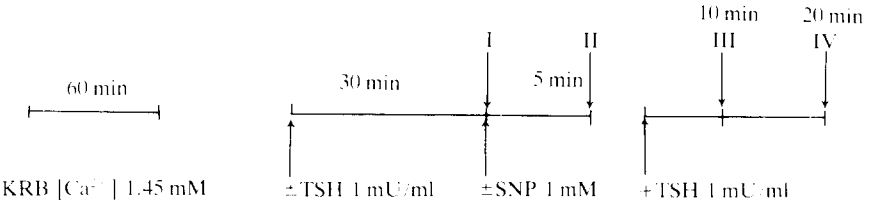
Table 3. Sodium nitroprusside (SNP) (1 mM) does not decrease cyclic-AMP accumulation in slices stimulated or not by TSH (1 mU/ml)



	No pulse			SNP pulse 1 mM (5′)		
	0 min	10 min	20 min	0 min	10 min	20 min
0	17 ± 7		17 ± 1	31 ± 4	25 ± 6	24 ± 8
TSH (1 mU/ml)	345 ± 20	302 ± 78	254 ± 92	417 ± 74	273 ± 10	303 ± 15

The slices were preincubated 1 hr and incubated in a medium containing 1.45 mM calcium. After the preincubation, the slices were first incubated 30 min in the presence or in the absence of TSH 1 mU/ml, then 5 min in the presence or not of sodium nitroprusside (SNP) (1 mM), then they were transferred in a fresh medium containing or not TSH (1 mU/ml). Caffeine (1 mM) was added 20 min before the end of the incubation. The results are expressed in pmoles of cyclic-AMP per 100 mg wet wt (±S.E.M.).

Table 4. Effect of sodium nitroprusside (SNP) (1 mM) on cyclic-GMP accumulation (A) and cyclic AMP accumulation in dog thyroid slices stimulated by TSH (1 mU/ml) (B)



PDE inhibitor	30 min (I) 0	30 min (I) TSH	TSH 30 min SNP 5 min (II)	Treatment TSH 30 min SNP 5 min TSH 10 min (III)	TSH 30 min SNP 5 min 20 min (IV)	TSH (30 + 5) min TSH 10 min (III)	TSH (30 + 5) min 20 min (IV)
A. Cyclic-GMP accumulation							
IBMX 0.1 mM	32 ± 10	31 ± 3	1901 ± 136	336 ± 12	176 ± 25	45 ± 12	50 ± 15
Ro 20-1724 0.1 mM	25 ± 2	29 ± 9	1534 ± 222	228 ± 68	116 ± 10	44 ± 3	34 ± 8
B. Cyclic-AMP accumulation							
IBMX 0.1 mM	45 ± 4	498 ± 108	543 ± 70	478 ± 126	494 ± 50	535 ± 58	397 ± 219
Ro 20-1724 0.1 mM	48 ± 12	1298 ± 255	1181 ± 372	1165 ± 281	1016 ± 388	1153 ± 172	1307 ± 86

The results are expressed in pmoles of cyclic-GMP per g wet weight (±S.E.M.) and in pmoles of cyclic-AMP per 100 mg wet weight (±S.E.M.). The phosphodiesterases inhibitors IBMX and Ro 20-1724 were present in the preincubation medium and in the incubation medium. PDE: cyclic nucleotide phosphodiesterase.

sodium nitroprusside did not relieve the inhibition by carbamylcholine of the TSH induced cyclic-AMP accumulation (Fig. 4).

DISCUSSION

Carbamylcholine enhances calcium influx, increases intracellular cyclic-GMP and decreases cyclic-AMP in TSH stimulated dog thyroid slices [2, 3]. The decrease in cyclic-AMP accumulation is caused by a stimulation of this nucleotide catabolism [4]. As a cyclic-GMP activated cyclic nucleotide phosphodiesterase has been demonstrated in thyroid, the hypothesis had been proposed that the action of carbamylcholine may be mediated by cyclic-GMP activation of this phosphodiesterase [7, 11]. However, several indirect arguments bear against the hypothesis that cyclic-GMP is the factor responsible for the negative regulation of cyclic-AMP accumulation [25].

We show here that agents which can be transformed to nitric oxide, as sodium nitroprusside [21], greatly enhanced cyclic-GMP accumulation in dog thyroid slices. These effects contrary to those of carbamylcholine take place in calcium depleted slices. Raising cyclic-GMP accumulation with these drugs did not depress TSH induced cyclic-AMP accumulation. In calf thyroid slices, Spaulding [26] has shown that other nitrogenous compounds, hydroxylamine and sodium nitrite, significantly

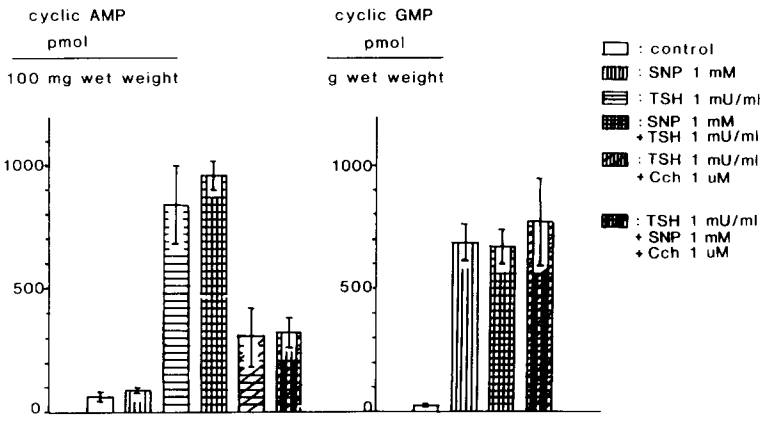


Fig. 4. Effect of sodium nitroprusside (SNP) (1 mM) on the inhibition by carbamylcholine of the cyclic-AMP accumulation in TSH stimulated slices.

reduced cyclic-AMP levels previously raised by TSH. However, the concentration of these nitrogenous compounds used was 20 mM compared to 1 mM in our experiments. Our results therefore suggest that elevating cyclic-GMP levels *per se* is not sufficient to decrease cyclic-AMP levels in dog thyroid slices. It could be argued that the nitrogenous compounds used in our experiments could both enhance cyclic-GMP accumulation and prevent cyclic-AMP decrease. However sodium nitroprusside did not inhibit the action of carbamylcholine.

In conclusion, we show here that increases in cyclic-GMP levels in dog thyroid slices do not depress cyclic-AMP accumulation. The carbamylcholine inhibition of cyclic-AMP accumulation can therefore not be explained by its enhancement of cyclic-GMP accumulation and a consequent stimulation of cyclic-GMP activated cyclic nucleotide phosphodiesterase.

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