INCREASED CYCLIC AMP-INDEPENDENT PROTEIN KINASE ACTIVITY IN THYROID TOXIC ADENOMAS

Y. MUNARI, J. ORGIAZZI and R. MORNEX

Groupe de Physiopathologie Endocrinienne, Hôpital de l'Antiquaille 69321 Lyon Cédex 1, France

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1. Introduction

Little is known so far on the mechanisms involved in the autonomous hyperfunction of thyroid toxic adenomas (TA). Because of its importance in cell growth, differentiation and function, the activity of the adenylate cyclase-cyclic AMP-protein kinase system has been investigated in TA. However, while the adenylate cyclase activity has been found generally hyperactive and hyperresponsive to TSH [1–3], no consistent pattern has been observed for the protein kinase activity [4]. We report here evidence for an elevated cyclic AMP (cAMP)-independent protein kinase activity (PKA) in the cytosols from TA as compared to normal thyroid tissue (N).

2. Material and methods

2.1. *Patients*

Samples of thyroid tissue were obtained at surgery from 8 patients operated on for a TA. None of these patients had received antithyroid drugs prior to surgery. Diagnosis of TA had been assured by a thyroid scintiscan showing radioactivity only in the nodule area. Each patient showed some degree of clinical hyperthyroidism. For comparison, samples of normal thyroid tissue (N) were obtained from 8 euthyroid patients operated on for a cold nodule. These samples showed no gross macroscopic abnormality and were later confirmed to be normal by histological examination. Plasma T4 and T3 resin uptake were 7.1 ± 0.6 μg/dl and 25.4 ± 0.4% in the euthyroid and 12.1 ± 1.9 μg/dl and 33.3 ± 2.6%, in the TA patients ($p<0.05$). Plasma T3 was 132 ± 7 ng/dl in the euthyroid and 235 ± 56 ng/dl in the 3 hyperthyroid patients so tested ($p<0.05$).

2.2. *Tissue preparation*

Fresh thyroid samples were sliced at 0–4°C using a Stadie Riggs microtome. The slices were washed, 3 times in 10 min, in 5 mM phosphate, 2 mM EDTA buffer (pH 7.0), then in 0.25 M sucrose, 20 mM Tris–HCl, pH 7.4, (Tris sucrose) in which they were homogeneized first in 3 vol./wt tissue with a Waring Blendor (high speed, 4 periods of 30 s) and then further with an all glass motor-driven homogeneizer. The homogenates were filtered through 4 layers of gauze and centrifuged at 105 000 × *g* for 1 h. The pellets were washed twice in 5 ml Tris–sucrose, centrifuged at 105 000 × *g* for 2 h and finally resuspended in 4 ml 50 mM Tris–HCl, pH 7.2. The 105 000 × *g* supernatants and the washed pellets were stored in aliquots at −20°C until use.

2.3. *Protein kinase activity*

PKA was assayed in 25 μl aliquots of soluble and particulate material as in [5] in the presence of 50 μg histone IIA (Sigma), 0.7×10^{-5} M or, in some experiments varying amounts of non radioactive ATP ranging from 10^{-6}–10^{-3} M and 5×10^{5} cpm of [γ-^{32}P]ATP (0.5–3.0 Ci/mmol; Amersham), without or with various doses of cAMP, in final vol. 225 μl. Preliminary experiments had shown that in these conditions the PKA of the soluble and particulate fractions from both TA and N tissues was linear for amounts of protein up to 50 μg/tube and for more than 20 min incubation. NaF, 10 mM was added only for the assay

of the particulate material. Blank values determined in the presence of 50 μg bovine serum albumine (BSA) instead of histone were subtracted from the experimental values. Blank values were not different in TA and N and amounted to less than 10% experimental activity.

The cAMP-dependent protein kinase heat-stable inhibitor was purified from 250 g rat brain as in [6]. The amount of inhibitor to be used in the assay was determined in preliminary dose–effect experiments on basal as well as maximally cAMP-stimulated PKA of various thyroid tissues. While an average basal PKA of 4 pmol phosphorus (P) transferred to histone per 10 min was maximally inhibited by 6 μg inhibitor/tube, 26 μg were necessary to abolish the 2–4-fold PKA stimulation elicited by 10^{-5} M cAMP. Therefore, we routinely used 26 μg inhibitor/assay tube in all experiments.

2.4. *Assays*

DNA assay was performed as in [7] modified [8] with calf thymus DNA (Sigma) as the standard. The DNA content was 1.53 ± 0.22 μg/mg and 1.14 ± 0.08 μg/mg tissue (wet wt) for N and TA, respectively, a difference not significant. The protein content of the cytosols, expressed per 100 mg wet wt, was 3.37 ± 0.17 mg and 2.08 ± 0.17 mg and that of the particulate fraction 2.03 ± 0.33 mg and 2.27 ± 0.11 mg for N and TA, respectively, as determined [9] with BSA standard. These differences were not significant. The cAMP concentration was assayed in thyroid cytosols after 0.3 M perchloric acid precipitation and purification through a Biorad AG 50 W × 4 column eluted with 0.1 N HCl. The fractions containing the cAMP were pooled, lyophilized and assayed as in [10]. The recovery of the extraction was 95–99% (10 expts). The cAMP concentrations in cytosols from N and TA tissues were 7.7 ± 1.1 pmol/mg DNA and 9.9 ± 2.8 pmol/mg DNA, respectively. This difference was not significant.

3. Results

In TA, basal soluble PKA was twice as high as in N (table 1). This was observed whether PKA was expressed per μg DNA (table 1) or per mg proteins (TA = 249 ± 30; N = 108 ± 18 pmol P transferred/10 min; $p<0.01$). In the presence of 10^{-5} M cAMP the PKA was also significantly greater in TA than in N, although the difference was less marked than for the basal activity. Therefore, the percent increase over basal elicited by 10^{-5} M cAMP was within normal limits. It is of interest to note that in both types of tissues half maximal activation of PKA occurred at similar concentration of cAMP, approx. 10^{-7} M. Basal PKA was also measured in another experiment, in the presence of varying amounts of ATP (table 2). The difference between TA and N was observed up to a maximal concentration ATP (10^{-3} M). In contrast,

Table 1
Soluble and particulate PKAs in extracts from TA and N thyroid tissues

	Cytosol			Particulate fraction		
	Basal (A)	cAMP 10^{-5} M (B)	B/A	Basal (A)	cAMP 10^{-5} M (B)	B/A
TA (8)	4.43[a] ± 0.37	7.88 ± 0.44	1.82 ± 0.09	0.48 ± 0.12	0.69 ± 0.12	1.54 ± 0.17
N (8)	2.26 ± 0.33	5.36 ± 0.76	2.42 ± 0.26	0.44 ± 0.11	0.66 ± 0.18	1.46 ± 0.12
P	< 0.001	< 0.02	n.s.	n.s.	n.s.	n.s.

[a] Mean ± SE : average of values obtained in 2 different experiments performed at several months interval

PKA is expressed as pmol P transferred to histone/μg DNA/10 min incubation
( ) no. cytosols tested

Table 2
Measurement of basal cytosolic PKA in the presence of increasing amounts of ATP

	ATP			
	10^{-6} M	10^{-5} M	10^{-4} M	10^{-3} M
TA (4)	0.63 ± 0.16[a]	4.21 ± 0.8	13.25 ± 3.07	16.81 ± 3.83
N (6)	0.50 ± 0.05	2.39 ± 0.21	5.59 ± 0.64	7.10 ± 1.66
P	n.s.	< 0.05	< 0.02	< 0.05

[a] Mean ± SE of PKA (pmol P transferred/μg DNA/10 min)

( ) no. cytosols tested

in the particulate fraction obtained from TA, both basal and stimulated PKA expressed per μg DNA, were normal. When expressed per mg particulate proteins, they were slightly subnormal, although not significantly (TA = 20.8 ± 3.8; N = 31.3 ± 6.4 pmol P transferred/10 min). Similarly, the stimulability ratio was normal in the particulate fraction.

In order to study further the abnormality of PKA in TA, the soluble enzyme activity from N and TA was assayed in the presence of cAMP dependent PKA inhibitor. Results are shown in table 3. The inhibitor suppressed an activity amounting to 1.1 and to 0.59 pmol P transferred/μg DNA/10 min in TA and N, respectively. This represented a decrease of about 30% for both. In both kinds of tissue extracts, the inhibitor completely antagonized the effect of 10^{-5} M cAMP showing the effectiveness of this dose of inhibitor to be sufficient. Despite this potency of the inhibitor, the remaining basal PKA in TA was more than twice the normal value ($p<0.01$). Although the results shown in table 2 might indicate a slight increase of the basal cAMP-dependent PKA in TA, they clearly show a marked increase of the PKA was not suppressed by the inhibitor. Therefore, assuming that residual PKA in the presence of inhibitor is cAMP independent, one can conclude that the soluble cAMP-independent PKA is clearly supranormal in TA.

4. Discussion

Our data indicate a greater soluble PKA in TA than in N. This difference was observed repeatedly in 3 different assays performed several months apart on frozen aliquots of each thyroid cytosol. This suggests that storage in the frozen state at −20°C did not

Table 3
Effect of the heat-stable protein kinase inhibitor on basal and stimulated cytosolic PKA

	No inhibitor		Plus inhibitor (26 μg/tube)	
	No cAMP	cAMP 10^{-5} M	No cAMP	cAMP 10^{-5} M
TA (8)	4.27 ± 0.41[a]	9.27 ± 0.63	3.17 ± 0.45	3.89 ± 0.46
N (8)	1.97 ± 0.19	5.60 ± 0.66	1.38 ± 0.14	1.67 ± 0.15
P	< 0.001	< 0.01	< 0.01	< 0.001

[a] Mean ± SE of PKA (pmol P transferred/μg DNA/10 min)

( ) Number of cytosols tested

affect the PKA significantly. The difference in PKA between TA and N was observed at low as well as high ATP concentration. Moreover, the ATP concentration giving half-maximal PKA was similar in both types of thyroid tissue. The difference in PKA was more marked in the absence than in the presence of maximal cAMP concentration, so that the cAMP stimulation ratio tended to be lower, although not significantly, in TA than in N. Such a difference could be explained by a higher cAMP content of the TA cytosols. However, it is very unlikely that the small and not significant cAMP increase observed in the TA cytosols could account for it. Therefore, our results strongly suggest the presence in the thyroid cells from TA of an increased soluble PKA. The observation of a normal stimulability ratio by cAMP in TA seemed to indicate that the increased PKA was not due to an augmentation of the cAMP-dependent PKA. However, in order to elucidate further this point, we studied the soluble PKA in the presence of the thermostable cAMP-dependent inhibitor. The inhibitor did not abolish the difference between PKA from N and TA. Furthermore, in other experiments (manuscript in preparation) we also observe a normal capacity and affinity of the cAMP-binding activity in TA as measured by equilibrium dialysis. These observations suggest that in cytosols from TA the cAMP-dependent PKA was normal and that the extra PKA was mainly of the cAMP-independent type. It is interesting to note that the abnormality was restricted to the soluble enzyme, since the particulate activity was normal.

The mode of expression of soluble enzyme activities in the thyroid is a sensitive point. Since thyroglobulin is the major soluble protein, referring to the amount of protein could be misleading. For this reason, results were expressed per unit of DNA. However, whatever the mode of expression, the results were similar. Indeed, we have found no difference in the DNA content expressed on a wet weight basis between N and TA. This is in keeping with a recent report in which no systematic alteration of the cellular/colloid ratio was observed in TA [4].

The presence in the thyroid tissue of both cAMP-dependent and -independent PKAs has been documented [11–13]. An increased activity of both enzymes after chronic treatment of rats by TSH was reported [13]. It is interesting to note that in TA, despite the low levels of circulating TSH, only the cAMP-independent PKA was clearly elevated. Since the respective involvement of the cAMP-dependent and -independent protein kinases in the thyroid function regulation processes is still poorly understood, the significance of this finding is not clear. It should be mentioned that in contrast to the present observation in TA, we reported on the absence of abnormality of the PKA in Graves' disease thyroid tissue [5].

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