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LACK OF ADENYLATE AND GUANYLATE CYCLASES RESPONSIVENESS TO HORMONES IN A SPONTANEOUS MURINE THYROID TUMOR

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SUMMARY

Animals with tumors were obtained from Dr. ZAJDELA and belong to sublines (XVIInc/Z/E) in which some individuals (TT) developed after 15 months thyroid tumors weighing between 150 and 1200 mg. Hyperplasia affects thyrocytes which do not present a follicular structure. The purpose of our work was to assay the action of various effectors on the adenylate and guanylate cyclase system in vitro. The following results have been obtained : the cyclic-AMP content of tumor tissue is not raised either by TSH or PGE_2. Nevertheless, TSH enhances the phosphatidylinositol phosphate turnover (phospholipid effect) as in normal tissue. This latter observation points at the existence of functional TSH receptors in tumor cells. The study of adenylate cyclase activity of the tumor homogenate shows the presence of this enzyme and its responsiveness to NaF and GppNHp. Unexpectedly, the cyclase is also sensitive to the stimulation by TSH.A tentative interpretation of these facts is that no component of the cyclase is missing, but that they are physically separated. The homogeneization allows the various components to interact productively.

A parallel study was devoted to cyclic-GMP. Carbamylcholine fails to increase the cyclic-GMP content of the tumor tissue, whereas it has the described phospholipid effect on phosphatidylinositol. Nevertheless, there is no deficiency in the guanylate cyclase activity, since nitroprusside enhances strongly the cyclic-GMP content of the tumor.

To conclude, the murine thyroid tumor presents a genetic alteration that results in the uncoupling of effector binding and catalytic stimulation of adenylate and guanylate cyclase.

This work aims at identifying a biochemical defect in relation with the presence of a genetic spontaneous tumor of the murine thyroid. The animals bearing tumors belong to sub-lines (XVII nc/Z/E) obtained

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Abbreviations: cyclic AMP, adenosine 3': 5'-monophosphate; cyclic GMP, guanosine 3': 5'-monophosphate; GPP (NH)p, guanyl-5'-yl imidodiphosphate.

Enzymes: Adenylate cyclase (EC 4.6.1.1); guanylate cyclase (EC 4.6.1.2)

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by imbreeding the progeny, where some individuals (TT), developed thyroid tumors when 15 months old. The tumors weigh between 150 mg and 1200 mg and are accompanied with leanness . Hyperplasia concerns thyrocytes which do not exhibit a follicular structure.

The stimulation of the thyroid by thyrotropin (TSH) implicates cyclic-AMP as second messenger (I). Nevertheless it is not an exclusive mode of action and some effects of TSH may be cyclic-AMP independent.

This independence is well established for the "phospholipid effect" (2,3) which is the increased turnover of the phosphoryl-inositol moiety of phosphatidylinositol (4). Two discrete sets of receptors for TSH have been described in the thyroid (5) but it is still unknown whether one is related to the adenylate cyclase stimulation and the other to the phospholipid effect. Moreover, the thyroid presents muscarinic receptors ; their occupancy by acetylcholine or carbamylcholine (CCh), induces the "phospholipid effect" (6) and the stimulation of guanylate cyclase (7).

As a first approach we decided to compare the phospholipid effect and the stimulation of adenylate and guanylate cyclases, by TSH, CCh and other stimulators, in normal and tumor tissues.

Our results suggest that in tumor cells, there are receptors for TSH uncoupled with adenylate cyclase and receptors for CCh uncoupled with guanylate cyclase.

MATERIALS AND METHODS

Material. The tumorous mice (TT) belong to sublines (XVII nc/Z/E) selected by Dr. F. ZAJDELA in Institut du Radium, Orsay, France. Swiss mouse normal glands were used as control.

Thyrotropin (TSH) was a gift from the Endocrinology Study Section (N.I.H. Bethesda). Prostaglandin E_2 (PGE_2) was kindly donated by Dr. J. Pike (Upjohn, Co). Purchases were made from the following sources : carbamylcholine (CCh) from Calbiochem, theophylline from Sigma, sodium nitroprusside and ethylene-diamine tetraacetic acid (E.D.T.A.) from Merck, cyclic-AMP from Boehringer, ^{32}P-phosphate (P_i) (10 mCi/ml) from C.E.N. Saclay - France - 2'O-succinyl (^{125}I) iodo-

tyrosyl-methylester of 3'5' AMP and 2'O-succinyl (^{125}I) iodo-tyrosyl-methylester of 3'5' AMP were supplied by Prof. Delaage, Marseille - France -.

Assay of cyclic nucleotides. Three normal mice thyroid lobes or an equivalent amount of thyroid tumor were routinely preincubated 30 min. at 37°C in 0.5 ml Krebs-Ringer bicarbonate buffer (0.1 M, pH 7.4) containing glucose (1 mg/ml). Then the tissues were transferred into the same medium (1 ml) containing bovine serum albumin (1 mg/ml) and various effectors : theophylline, TSH, PGE_2,CCh, Na nitroprusside. The incubation was ended by the addition of 1 ml of perchloric acid (I N) in an ice-bath. The tissues were homogeneized in a micropotter (Duall Kontes AA). Cyclic nucleotides (cyclic-AMP and cyclic-GMP) were assayed in the perchloric supernatant according to the radioimmunological method of Cailla et al. (8,9) except that the filtration on Millipore filters (HAWP 0.45 µ) was substituted for equilibrium dialysis.

Phospholipid-effect measurement and expression. Preincubation of the tissues was effected under the same conditions as for the cyclic nucleotides assay. Incubation was performed in the same medium during 90 min. in the presence of ^{32}P-phosphate (Pi) 10 µCi. After chilling in liquid nitrogen, the tissues were homogeneized in the incubation media. Phospholipids were extracted according to Folch et al. (10). and Bligh and Dyer (11) though adapted to small volumes. The counting of an aliquot of the chloroformic phase gave the total incorporation of the label in the phospholipids. The remainder was analysed according to Marinetti and Stotz (12). The phospholipid effect is expressed as the ratio of the radioactivity incorporated in the phosphatidylinositol (PI) and the phosphatidylcholine (PC).

Adenylate cyclase assay. Normal and tumor tissues were homogenized in Krebs-Ringer bicarbonate buffer. Aliquots (20 µl, 0.25 mg protein) were incubated 10 min. at 23°C in a final volume of 60 µl containing 50 mM TrisHCl, pH 7.6, 3 mM $MgCl_2$, 0.5 mM (^{32}P) ATP (10^6cpm), 1 mM E.D.T.A., 1 mM cyclic-AMP, 25 mM phosphocreatine and 1 mg/ml creatine phosphokinase. The various effectors were present in the following concentrations : NaF (5 mM), TSH (20 mU/ml), guanyl-5'-yl imidodiphosphate (Gpp(NH)p) (0.1 mM). Incubation was ended by addition of 200 µl HCL (0.5 M) followed by heating for 6 min. at 100°C. After neutralization with 200 µl imidazole (1.5 M) the supernatants were applied to alumina columns. Cyclic-AMP was eluted with HCl (50 mM)-imidazole (0.1 M) buffer (13) and counted.

Other methods. In all the experiments the denatured pellets were solubilized in NaOH and protein estimation was performed according to Lowry et al. (14) using bovine serum albumin as a standard.

Values are given as means + S.E. of triplicate determinations. The experiments were reproduced 2 or 3 times with very similar or identical results.

RESULTS

Figure 1 (a,b) - shows the cyclic-AMP concentrations in normal thyroid lobes following exposure to TSH or PGE_2 stimulation. These values have to be compared to those obtained when the same TSH and PGE_2 concentrations are incubated with tumor fragments. The absence of stimulation observed in the latter case could be attributed to the

in a majority of the transformed cells resulted in the arrest of these cells in the S phase of the cell cycle (8, 9). Recently, Mamont et al (10) have shown that α-difluoromethyl ornithine (DFMO), a catalytic irreversible inhibitor of ornithine decarboxylase (11), decreased the concentrations of putrescine and spermidine and caused a rapid inhibition of cell growth in rat hepatoma and mouse leukemia cells in culture. In addition, DFMO has been shown to inhibit the growth of experimental tumors in mice (12, 13). In the present study, we have investigated the effect of DFMO on the growth and cell cycle traverse of HeLa cells. The results of our study indicate that DFMO causes a rapid inhibition of HeLa cell growth as a results of depletion of intracellular levels of putrescine and spermidine, and that it arrests a majority of the cells in S phase. Further, we found that a sequential administration of DFMO and Ara-c, an S phase-specific drug, results in a synergistic antiproliferative effect on HeLa cells.

MATERIALS AND METHODS

Cells and cell synchrony. HeLa cells were routinely grown as monolayer cultures as described earlier (14). These cells have a cell cycle time of 22 hr, consisting of 10.5 hr of pre-DNA synthetic (G2) period, and 1.0 hr of mitosis (15). To obtain mitotic cells for cell fusion experiments, an exponentially growing culture was partially synchronized by a single thymidine block that was followed by N_2O block for 9 hr, and the separation of mitotic cells by selective detachment (16). Cells thus harvested had a mitotic index of about 98%.

Cell kinetics. HeLa cells were plated in 150-mm culture plates at a cell density of 2.5×10^5 cells per plate. The cells were grown either in the presence or absence of DFMO (2.5 mM) at 37°C. At appropriate incubation periods, cells were collected by trypsinization and counted, and a portion of the cells (2×10^6 cells) was used to determine cell cycle kinetics of both the populations.

To find where the DFMO treated cells were blocked, we applied the technique of premature chromosome condensation (17). This method, which involves the Sendai virus-mediated fusion between mitotic and interphase cells, makes it possible to determine the position of an interphase cell in the cell cycle on the basis of the morphology of its prematurely condnesed chromosomes (PCC). Recently, this method has been used for cell cycle

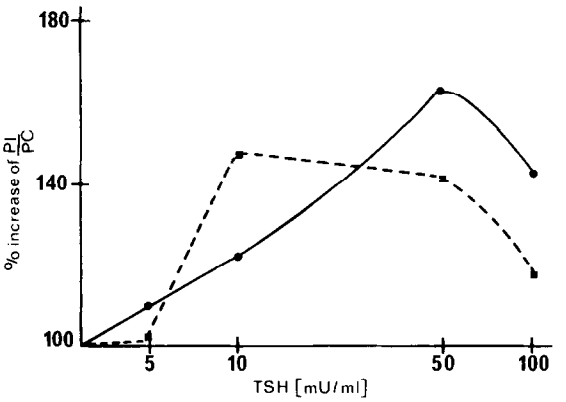


Figure 2. The "phospholipid effect" of TSH on tumorous (——) and normal (----) thyroid tissue. Thyroid tumor fragments and normal lobes were incubated 90 min. in the presence of labelled Pi and various TSH concentrations. At the end of the incubation, phsopholipids were extracted and analysed as described in Methods.

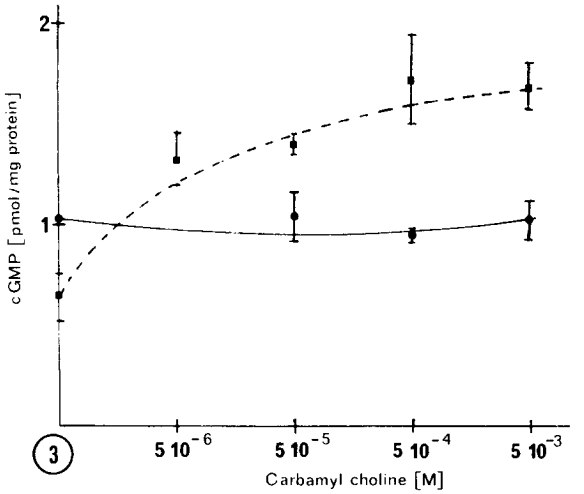
To localize more precisely the defect it was necessary to ascertain the sensitivity of the cyclase. The adenylate cyclase activity of homogenates of normal and tumor tissues was measured after stimulation by TSH, Gpp(NH)p and fluoride. As expected, normal tissue adenylate cyclase is stimulated by the three agonists (Table 1). In tumor homogenate, adenylate cyclase is stimulated by fluoride and Gpp(NH)p. More surprising is the slight stimulation evoked by TSH.

The same kind of experiments were performed in order to test the sensitivity of guanylate cyclase by carbamylcholine. It is clear that no stimulation of cyclic-GMP accumulation can be observed in tumorous tissue under concentrations of agonist effective in normal tissue (Figure 3). Nevertheless, the existence of functional receptors binding CCh is demonstrated by the phospholipid effect (Figure 4). This effect is maximal in tumor tissue with CCh 5 X 10^{-6}M, at variance with the response of normal tissue. The functional character of guanylate cyclase can be demonstrated with nitroprusside (15) which produces a five-fold stimulation of cyclic GMP accumulation (Table 2).

Table 1.

	NORMAL TISSUE		TUMOR TISSUE	
	pmol c-AMP/I0min./mg protein	% stimulation	pmol c-AMP/I0min/ mg protein	% stimulation
Base	30.63 ± 0.55		95.I3 ± 0.35	
NaF 5 mM	69.I0 ± 3.03	+ I25.56	I5I.73 ± I2.05	+ 57.82
Gpp (NH)p 0.I mM	I07.27 ± 5.25	+ 250.I4	23I.55 ± 9.23	+I40.85
TSH 20 mU/ml	37.84 ± I.6I	+ 23.54	II3.43 ± 4.52	+ I7.98
Gpp (NH)p 0.I mM + TSH (20 mU/ml)	II4.85 ± 2.I3	+ 274.89	250.02 ±2I.93	+I60.06

Activation of adenylate cylase of normal and tumorous tissue homogenate by fluoride, Gpp(NH)p and TSH. Tissue homogenates (0.25 mg prot.) were incubated 10 min. at 23°C with the various effectors and adenylate cyclase activity was measured as indicated in Methods.



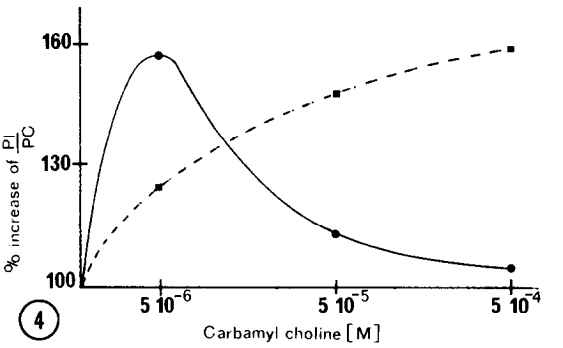


Figure 3. Cyclic-GMP levels in normal (----) and tumorous (——) thyroid tissue under carbamylcholine stimulation. The tissues were incubated 15 min. at 37°C in the presence of the effectors and cyclic-GMP was assayed as indicated in Methods.

Figure 4. The "phospholipid effect" of carbamylcholine on tumorous (——) and normal (----) thyroid tissue. Thyroid tumor fragments and normal lobes were incubated 90 min. in the presence of labelled Pi and various CCh concentrations. At the end of the incubation phospholipids were extracted and analysed as described in Methods.

Table 2.

	pmol c-GMP/mg protein
Basal	0.355 ± 0.020
CCh 5 x 10^{-5} M	0.334 ± 0.060
Nitroprusside 10 µM	1.570 ± 0.120
Nitroprusside 1 mM	1.620 ± 0.040

Cyclic-GMP levels in tumorous thyroid tissue stimulated by nitroprusside.
The tissue fragments were incubated 5 min. at 37°C in the presence of theophylline 10^{-2} M with the effectors. Cyclic-GMP was assayed as indicated in Methods.

DISCUSSION

The absence of stimulation of the adenylate cyclase activity by TSH in tumor tissue, whereas the "phospholipid effect" occurs, shows the presence of functional receptors and suggests their uncoupling from the adenylate cyclase. This situation is not similar to that

described by Macchia et al. (16) who studied a transplantable rat thyroid tumor and concluded that an alteration of the receptor subunit occurs.

The functional character of the cyclase is demonstrated by its stimulation in the presence of fluoride or Gpp (NH) p. Neither the catalytic subunit of the cyclase, nor the regulatory protein (s) (G/F according to Gilman (17)) are lacking and they interact productively when fluoride or Gpp (NH) p are present. The most striking and unexpected result was the stimulation by TSH of the adenylate cyclase in the tumor homogenate. This stimulation which is weak but significant, is comparable to that obtained in normal tissue homogenate in the absence of added GTP.

A similar situation was reported long ago by Ney et al. (18) in an adrenal tumor but at that time was given no explanation. To day, to explain the recovery of the adenylate cyclase sensitivity to TSH after homogeneization, it is tempting to speculate that in the tumor tissue the coupling subunit (G/F) is not bound to the plasma membrane and that the cell desintegration allows the reconstitution of a hormone sensitive system. This hypothesis is supported by observations made in other cell-types (19). Detergent solubilized G/F and a membrane bound catalytic subunit can interact and be subjected to regulation by hormones without a stable association of G/F with the membrane. Such speculations can be tested in reconstituted acellular systems.

We cannot conclude so far whether the phospholipid effect is part and parcel of the adenylate cyclase system or is located on another pathway. The advantage provided by the study of the phospholipid effect in our system is to show that its amplitude is comparable in normal and tumor tissues. Cells from the tumor, as a whole, must have retained receptors and a biological response. This finding

allows to discard the possibility that only a few cells in the tissue mass have an adenylate cyclase responsive to TSH, the others being undifferentiated. This situation could explain why the increment in cyclic-AMP production by responsive cells is occulted by the background due to the undifferentiated cells.

Uncoupling extends to guanylate cyclase activity. This latter statement is supported by the nitroprusside action. It is not possible to speculate further because the intimate mechanism of guanylate cyclase activation is still unknown.

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REFERENCES

1. For a review on the subject, see Dumont, J.E. (1971) Vitam. Horm. 29, 287-412.
2. Jacquemin, C., & Haye, B. (1970) Bull. Soc. Chim. Biol. 52, 153-165.
3. Scott, R.W., Freinkel, N., Klein, J.H., & Nitzan, M. (1970) Endocrinology 87, 854-863.
4. Scott, T.W., Jay, S.M., & Freinkel, N. (1966) Endocrinology 79, 591-598.
5. Pekonen, F., & Weintraub, B.D. (1978) Endocrinology 103, 1668-1667.
6. Altman, M., Oka, H., & Field, B.J. (1966) Biochim. Biophys. Acta 116, 586-588.
7. Yamashita, K., & Field, B.H. (1972) J. Biol. Chem. 247, 7062-7066.
8. Cailla, H.L., Racine Weisbuch, M.S., & Delaage, M.A., (1973) Anal. Biochem. 56, 394-407.
9. Cailla, H.L., Vannier, C.J., & Delaage, M.A. (1976) Anal. Biochem. 70, 195-202.
10. Folch, J., Less, M. & Sloane Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
11. Bligh, E.G., & Dyer, W.J. (1959) Can. J; Biochem. Physiol. 37, 911-917.
12. Marinetti, G.V. & Stotz, E. (1956) Biochim. Biophys. Acta 21, 168-174.
13. White, A.A. (1974) in Methods in Enzymol. 38c, 41-46. (Hardman, J.G. & O'Malley, B.W. eds) Academic Press, New-York.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

15. Katsuki, S., & Murad, F. (1977) Mol. Pharm. 13, 320-341.
16. Macchia, V., Tamburrini, O., & Pastan, I. (1970) Endocrinology 86, 787-792.
17. Ross, E.M., Howlett, A.C., Ferguson, K.M., & Gilman, A.G. (1978) J. Biol. Chem. 253, 6401-6412.
18. Ney, R.L., Hochella, N.J. , Grahame-Smith, D.G., Dexter, R.N. & Butcher, R.W. (1969) J. Clin. Invest. 48, 1733-1739.
19. Howlett , A.C., Sternweis, P.C., Macik, B.A., Van Arsdale, P.M. & Gilman, A.G. (1979) J. Biol. Chem. 254, 2287-2295.