STIMULATION BY THYROTROPIN OF HORSE THYROID PLASMA MEMBRANES ADENYLATE CYCLASE : EVIDENCE OF COOPERATIVITY

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SUMMARY

Horse thyroid plasma membranes were prepared by partition in an aqueous two phases system. The membrane fraction was enriched in adenylate cyclase and only slightly contaminated with mitochondria and lysosomes. Adenylate cyclase activity was stimulated by TSH and PGE_1. The TSH stimulatory effect was nearly immediate and occured in the same range of concentrations that activates intact cell metabolism. It was potentiated by GTP and ITP. A quantitative analysis of the data suggests that activation of thyroid adenylate cyclase by TSH is a cooperative process.

There is now much evidence that thyrotropin exerts its effects on thyroid gland through activation of the adenylate cyclase system (1). In particular, TSH has been shown to stimulate the adenylate cyclase present in thyroid homogenates and membrane preparations (2,3,4,5).
However, in no system so far investigated did the hormone activate the enzyme in the range of concentrations which elicit its stimulatory effects on intact cells. Horse thyroid plasma membranes prepared by partition in an aqueous two phases system (6,7) are

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enriched in an adenylate cyclase activity which is highly sensitive to TSH.

MATERIALS AND METHODS

Cyclic AMP, ATP, phosphocreatine, creatine kinase (75units/mg), p-nitrophenylphosphate were purchased from Sigma Chemical Co. α^{32}P-ATP (1Ci/mmole) was obtained from the Radiochemical Centre Amersham, ^{3}H-labeled cyclic AMP (12.7Ci/g) from New England Nuclear. Bovine thyrotropin was obtained from Armour and from the NIH.

Horse thyroid glands were collected in a local slaughterhouse just after killing and immediately put in a ice cold standard medium (sucrose 0.25M, Tris-HCl pH 7.4, 20mM). They were then rapidly trimmed free of fat and connective tissue, sliced with a Stadie Riggs microtome, chopped with a Mac Ilwain slicer and homogenized with a motor driven teflon glass homogenizer, by 4 strokes at a low speed. This preparation was filtered through a teflon sieve and centrifuged at 600g for 10 minutes at 4°C. A polymer aqueous two phases system (6,7) was prepared by mixing 190ml of Dextran 1500 (Pharmacia) (22% W/V), 100ml polyethylene glycol 6000 (BDH) (30% W/V), 200ml tris-HCl 0.2M, pH 7.4 and 100ml bidistilled water in a separatory funnel. The mixture was allowed to settle for 48 hours at 4°C. The 600g pellet was resuspended in the top phase and an equal volume of bottom phase was then added.
This mixture was centrifuged in a SW 25.1 Sorvall RC_2 rotor for 10 minutes at 11,500rpm (12,000g). The membranes were then collected at the interface of the two phases system. The whole preparation took less than three hours.

The membranes were carefully resuspended to appropriate dilutions in the standard medium and assayed for adenylate cyclase for 10 minutes at 30°C. The incubation medium (200µl) contained 50mM tris-HCl pH 7.4, 2mM cAMP, 5mM $MgCl_2$, 3mM ATP, 25mC/lATP-αP^{32}, 0.1% crystalline bovine serum albumin, 10mM creatine phosphate, 300mg/l crystalline rabbit muscle creatine

kinase. At the end of the incubation, the test tubes were transferred to an ice bath and at the same time 100ul of an ice cold solution containing cyclic AMP 20mM, ATP 50mM and ^{3}H cyclic AMP 20000cpm were added to each of them. Cyclic AMP was then isolated, in one step, by elution on dry neutral alumina columns(8): the eluate was diluted in Bray's solution, H^3 and P^{32} activities were counted in a liquid scintillation counter. The recovery of cyclic AMP was approximately 80% with less than 2.10^{-5} ATP contamination.

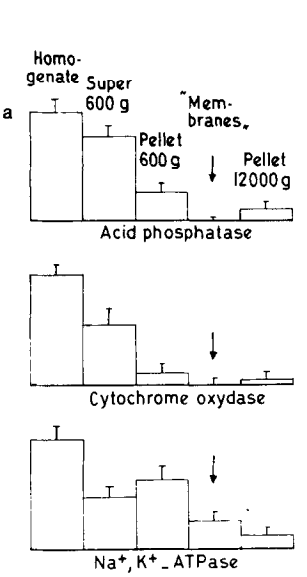
The results were expressed as picomoles cAMP formed in 10 minutes per mg membrane protein. Protein concentration (9), acid phosphatase (10); Na^+, K^+ dependent ATPase (11) and cytochrome C oxydase (12) were assayed by standard methods. Assays were always performed in duplicate or triplicate. Data were fitted by the least square method, using a CDC 6040 computer.

RESULTS

The fraction collected at the interface of the aqueous two phases system was only slightly contaminated with lysosomes and mitochondria as judged by the low activity of acid phosphatase and cytochrome C oxydase, respectively (figure 1a). It contained Na^+, K^+ dependent ATPase activity and was enriched in adenylate cyclase (figure 1b).

This adenylate cyclase activity was stimulated by TSH and PGE_1. The TSH effect began at a concentration of 0.03mU/ml and was half maximal at a concentration of 1.2mU/ml (mean of 6 experiments) or 1.10^{-9}M, if we assume that pure TSH has an activity of 40 I.U./mg (13) (figure 2). Maximally effective TSH concentrations stimulated adenylate cyclase activity by a factor of 5.0 (mean of 5 experiments). PGE_1 had a half maximal stimulatory effect at the concentration of 0.02µg/ml.

Adenylate cyclase was almost immediately fully activated by TSH (figure 3). Accumulation of cAMP in response to low TSH concentrations did not



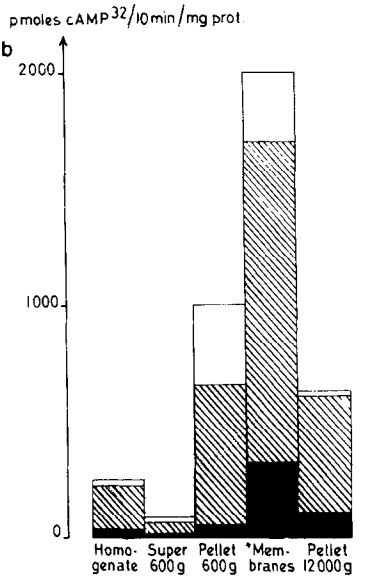


Figure 1a : Subcellular distribution of the total activity of different enzyme markers, as a fraction of their activity in the crude homogenate.

Figure 1b : Subcellular distribution of adenylate cyclase specific activity.

: basal activity

: TSH (20mU/ml) stimulated activity.

: NaF (10mM) stimulated activity.

decay with time up to 25 minutes, which suggests that the hormone was not significantly inactivated during the incubation with membranes. Basal, as well as TSH stimulated, adenylate cyclase activities were proportional to the membrane protein concentration (in the range 0.5-2.5mg/ml).

As previously shown (14), ITP activated adenylate cyclase and potentiated the TSH stimulatory effect; GTP had a similar effect, but to a lesser extent. The maximal effect of ITP was reached at a concentration of 100μM.

A Scatchard plot of the relationship between

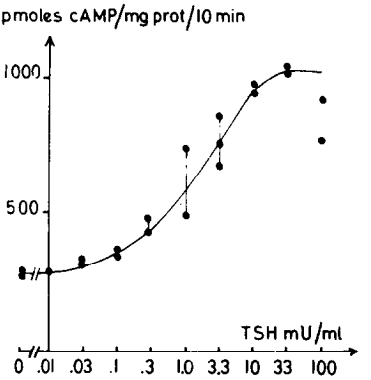


Figure 2 : Relation between TSH concentration and adenylate cyclase activity.

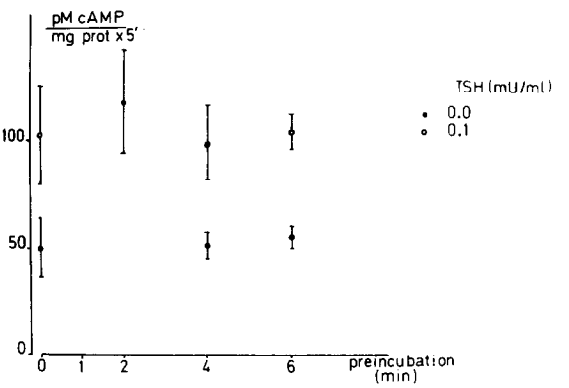


Figure 3 : Membranes were preincubated with or without TSH : their adenylate cyclase activity was then assayed for 5 minutes.

TSH concentration and adenylate cyclase activity was hyperbolic, indicating negative cooperativity (15,16). In the presence of ITP, the plot was modified : it then exhibited a maximum and a downward concavity, which is typical of positive cooperativity (17) (figure 4).

DISCUSSION

Using the conventional but lenghtly method of Touster et al. (18), Matsuzaki et al. (19) had been

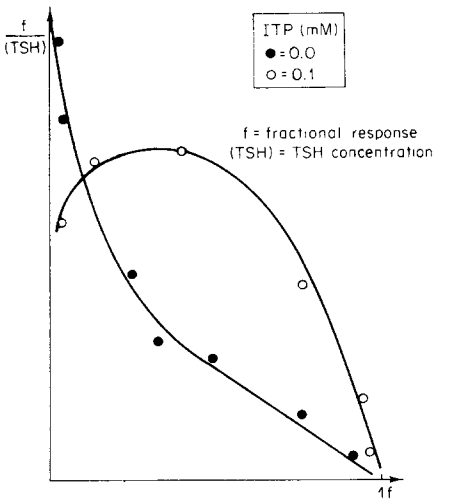


Figure 4 : Scatchard plots of the relationship between TSH concentration and adenylate cyclase response, expressed as a fraction of the maximal response, in the presence and absence of ITP.

unable to obtain horse thyroid plasma membranes responsive to TSH. Other authors prepared very purified thyroid membranes which responded to TSH, but only at very high concentrations, far above those which elicit the well known hormone effects on intact cells. For instance, Wolff and Jones working with bovine thyroid plasma membranes observed no activation of adenylate cyclase below 2mU/ml TSH (3). The recalculated data of Yamashita and Field (4) or the reported data of Kowalski et al. (5), obtained with similar preparations, do not show any stimulation below 5mU/ml. In fact, these authors routinely used concentrations as high as 20 to 200mU/ml TSH. On the other hand, most of TSH effects on thyroid intact tissue are half-maximal below 1mU/ml and maximal below 10mU/ml (1, 20-25).

With the convenient and rapid method of partition in a aqueous two phases system we have prepared a subcellular fraction of horse thyroid enriched in two enzyme markers for plasma membranes, Na^+, K^+ dependent ATPase and adenylate cyclase and only slightly

contaminated with mitochondria and lysosomes. This plasma membrane fraction contained adenylate cyclase activity that responded to a TSH concentration as low as 0.03mU/ml and was half-maximally stimulated by a concentration of 1.2mU/ml. These values correspond to the usual range of concentrations activating the intact tissue intermediary metabolism and specialized functions (1, 20-25).

Membrane bound adenylate cyclase is an almost ideal system for studying the molecular events associated with the expression of receptor functions : interaction with a specific hormone and generation of a response (26). Graphical representations and least square fitting of our data show that the process of adenylate cyclase activation by TSH is negatively cooperative in the absence of ITP and that it becomes positively cooperative in the presence of ITP. This negatively cooperative behavior could be explained by the existence of two different sets of receptor sites for TSH, as it has been reported for other hormones (27-29). It could also be accounted for if the receptors for TSH were cooperative structures, as recently suggested in the case of insulin (30). This last hypothesis fits well with the action of ITP this intracellular nucleotide would thus modulate the hormone receptor interaction at the outer face of the plasma membrane by switching it from negative to positive cooperativity.

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