THYROTROPIN-RELEASING HORMONE RECEPTOR: ITS PARTIAL PURIFICATION FROM BOVINE ANTERIOR PITUITARY GLAND AND ITS CLOSE ASSOCIATION WITH ADENYL CYCLASE*

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1. Introduction

A contribution of major interest in the field of neuroendocrinology has been the first and recent elucidation of the structure of one of the hypothalamic releasing hormones. The structure of thyrotropin-releasing hormone (TRH), the hormone which controls the activity of the thyrotropin-secreting cells in the anterior pituitary gland, has in fact been shown to be L-pyroglutamyl-L-histidyl-L-proline amide [1, 2].

Since there is strong evidence for a role of adenosine 3′,5′-monophosphate (cyclic AMP) as mediator of the action of the hypothalamic releasing hormones [3–10], and for a primary action of some polypeptide hormones by binding to a receptor closely related to adenyl cyclase, [11–14], it seemed important to attempt a purification of the receptor for TRH and to investigate the possibility of a close correlation of the subcellular distributions of ^{3}H-TRH binding and of adenyl cyclase activity.

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2. Materials and methods

Bovine anterior pituitaries were collected in local slaughterhouses and immediately brought to the laboratory in ice-cold Krebs-Ringer bicarbonate buffer, pH 7.4, containing 11 mM D-glucose. Plasma membranes were isolated by our modification of Neville's technique originally designed for the purification of plasma membranes from rat liver [15]. All procedures were carried out at 0–4°. Briefly, anterior pituitaries in 5 vol of buffer D (0.001 M $NaHCO_3$ –0.005 M mercaptoethanol) were treated for 30 sec at half-maximal speed in a Waring Blendor before homogenization with 15 strokes of a Glenco teflon-glass homogenizer having a clearance of 0.125 mm. 25 ml of buffer D were then added per g of original pituitary tissue and the dilute homogenate was stirred for 2 min at low speed before filtration through 4 layers of cheesecloth and centrifugation of the filtrate at 2,000 *g* for 30 min. The supernatant was removed by aspiration and discarded. The jelly-like pellet was resuspended with 2 strokes of the pestle in a loose-fitting homogenizer after addition of 4 ml of buffer D per g of pituitary tissue. The suspension was centrifuged in 50 ml conical tubes at 1,220 *g* for 20 min. The 1,220 *g* supernatant was aspirated and kept while the pellet was resuspended in 3 ml of buffer D per g of tissue, and the centrifugation at 1,220 *g* repeated once. The pellet was discarded and the 1,220 *g* supernatants pooled.

To obtain a first separation of plasma membranes

from the secretory granules also present in the 1,220 *g* supernatant, the fraction was sedimented at 30,000 *g* for 30 min. The supernatant was carefully aspirated and discarded, while the brownish top layer of the pellet, containing mostly membranes, was carefully removed for further purification. The white bottom layer of the 30,000 *g* pellet contained almost exclusively secretory granules. The brownish top layer was resuspended in 47.5% (w/v) sucrose in water (d = 1.18) and centrifuged at 95,000 *g* for 2.5 hr in a step-sucrose gradient containing layers of densities 1.14, 1.16, 1.18, 1.20 and 1.22. As shown by electron microscopy, enzyme markers, and the protein pattern obtained by polyacrylamide gel electrophoresis (G. Poirier, F. Labrie, S. Lemaire, G. Pelletier and A. Lemay, unpublished), the material sedimenting at the interface 1.14–1.16 consisted of pure plasma membranes, while the material accumulating at the interfaces of densities 1.18–1.20 and 1.20–1.22 consisted of rough microsomes. The secretory granules were quantitatively recovered in the pellet. This rather simple technique, when applied to anterior pituitary tissue, yields simultaneously plasma membranes, rough microsomes and secretory granules. These 3 fractions are of great interest for studies of the mechanism of action of the hypothalamic releasing hormones and of adenosine 3′,5′-cyclic monophosphate in the anterior pituitary gland.

3. Results and discussion

3.1. *Binding of ^{3}H-TRH to adenohypophyseal plasma membranes*

Plasma membranes isolated at the interface of sucrose densities 1.14 and 1.16 show a 40-fold increase of ^{3}H-TRH binding as compared to the total adenohypophyseal homogenate (fig. 1). The fraction sedimenting at 1.16–1.18 consists of plasma membranes contaminated to a variable extent by saccules originating probably from the Golgi complex and shows binding values intermediate between those of pure plasma membranes and of the rough microsomes which sediment at the interfaces 1.18–1.20 and 1.20–1.22. No binding can be measured in the pellet consisting of pure secretory granules.

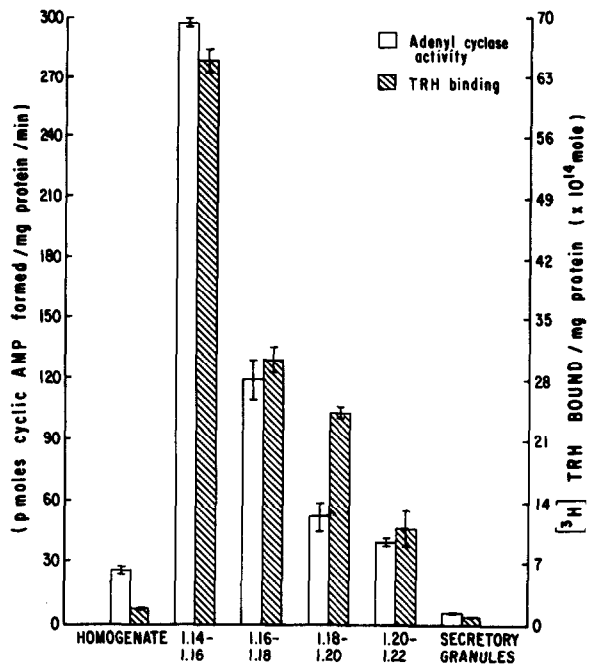

Fig. 1. Distribution of ^{3}H-TRH binding and fluoride-stimulated adenyl cyclase activity in adenohypophyseal membrane fractions. Membrane fractions were prepared as described in the text. Measurements of ^{3}H-TRH binding were performed as described [16]. In the standard assay, 50 μg of membrane protein were incubated in a final volume of 55 μl in buffer F (0.020 M sodium phosphate, pH 7.35–0.0075 M KCl–0.002 M $MgCl_2$) containing 5.0×10^{-8} M ^{3}H-TRH (55,000 cpm) at 0° for 40 min. The reaction was stopped by the addition of 1.0 ml of ice-cold buffer F and the medium immediately filtered with gentle suction through a Millipore filter with 3 successive washes with 2 ml of ice-cold buffer. After drying at 60° for 30 min, 10 ml of PPO-POPOP-toluene scintillation fluid were added and the radioactivity measured in a Packard liquid scintillation spectrometer. All assays were done in triplicate. Adenyl cyclase activity was determined according to Krishna, Weiss and Brodie [17]. The incubation was carried out for 10 min at 37° in medium containing 40 mM Tris-HCl, pH 7.4, 3.3 mM $MgSO_4$, 3.0 mM adenosine 3′,5′-monophosphate, 10 mM NaF and 1 mM ^{3}H-ATP.

It can also be seen in fig. 1 that the specific activity of adenyl cyclase measured in the presence of 10 mM NaF shows a pattern of distribution similar to the binding of ^{3}H-TRH. Maximal adenyl cyclase activity is in fact found in the plasma membrane fraction. The activity decreases progressively in the material sedimenting at higher densities in the

Table 1
Subcellular distribution of ^{3}H-TRH binding and adenyl cyclase activity in anterior pituitary gland.

Fractions	Adenyl cyclase activity (pmoles of cyclic AMP formed/mg protein/min)	TRH binding (femtomoles of ^{3}H-TRH bound/mg protein)
Total homogenate	70.6 ± 17	15.7 ± 0.1
Nuclei	113.6 ± 15.6	110.0 ± 7.1
Mitochondria	64.4 ± 7.3	65.6 ± 0.4
15 K (microsomes + secretory granules)	83.8 ± 5.3	84.5 ± 0.7
200,000 *g* Supernatant	7.9 ± 1.5	6.7 ± 0.6
Total microsomes	178.5 ± 13.0	207.5 ± 9.9
Rough microsomes	68.5 ± 3.1	46.1 ± 3.2
Smooth membranes	375.1 ± 12.6	526.0 ± 25.2

Subcellular fractions were prepared as described [5]. Measurements of ^{3}H-TRH binding and of adenyl cyclase activity were carried out as described in fig. 1.

sucrose gradient. A 10- to 15-fold purification of adenyl cyclase specific activity is usually observed in the plasma membrane fraction as compared to the total cell homogenate. Although ^{3}H-TRH binding and fluoride-stimulated adenyl cyclase activity are located almost exclusively in plasma membranes, the degree of purification of enzymatic activity is considerably lower than that of hormone binding. This finding is likely to be accounted for by the relative instability of the adenyl cyclase during preparation of the plasma membranes.

3.2. *Subcellular distribution of ^{3}H-TRH binding and adenyl cyclase activity*

Table 1 illustrates that both ^{3}H-TRH binding and adenyl cyclase activity are found almost exclusively in the particulate fractions, negligible amounts of either activity being found in the 200,000 *g* supernatant fraction. As previously observed for the membrane fractions separated by step-sucrose gradient (fig. 1), a good correlation exists in all subcellular fractions between TRH binding and adenyl cyclase activity. The appreciable level of hormone binding in the particulate fractions (nuclei, mitochondria and 15 K) could be accounted for by some contamination of these fractions by plasma membranes. Total microsomes contain high levels of both adenyl cyclase activity and TRH binding. Further purification of this fraction shows that both activities can be almost quantitatively recovered in smooth membranes showing the morphological and enzymatic properties of plasma membranes.

We have recently studied the properties of the interaction of ^{3}H-TRH with its receptor in adenohypophyseal plasma membranes [16] and have shown that the neurohormone interacts according to a bimolecular process with the cell membrane receptor. The interaction is reversible, highly specific and has a dissociation constant of about 3.0×10^{-8} M.

Preliminary studies in our laboratory suggest a direct interaction of the TRH receptor with adenyl cyclase in the plasma membranes of the anterior pituitary cell with resulting enhancement of enzymatic activity. The present demonstration of an almost exclusive location of the receptor for TRH in the plasma membrane which is also the main site of the adenyl cyclase in the anterior pituitary cell, as well as in the other mammalian tissues studied [18, 19], suggests strongly that the interaction of TRH with plasma membranes is the first step in hormone action and that this interaction is likely to be in functional relationship with the adenyl cyclase. The presented data also provide a means

for dissecting the overall action of TRH in the adenohypophyseal cell by studying the primary action of TRH, its interaction with the membrane receptor, instead of some later distant consequence of the primary TRH-receptor interaction. It could be argued that TRH could, in addition to its interaction with the plasma membrane, penetrate inside the cell and interact with some intracellular structure. However, our data on the subcellular distribution of ^{3}H-TRH binding (table 1) show that this potential intracellular binding could not represent more than a minor component. It should also be mentioned that the results which we have obtained so far indicate that binding of TRH shares many characteristics with the binding of larger peptides such as ACTH [12, 13] and glucagon [13, 14] to the plasma membranes of their target cells.

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