# Membrane Effects of Thyrotropin-Releasing Hormone and Estrogen Shown by Intracellular Recording from Pituitary Cells

Abstract. *The effects of thyrotropin-releasing hormone and 17β-estradiol on the electrical membrane properties of a prolactin-secreting pituitary cell line ($GH_3$/B6) were studied with intracellular microelectrode recordings. Of the cells tested, 50 percent were excitable and displayed calcium-dependent action potentials when depolarized. When injected directly on the membrane of an excitable cell, thyrotropin-releasing hormone and 17β-estradiol induced action potentials within 1 minute. The spiking activity was preceded by a progressive increase of the input resistance without any detectable change in the resting membrane polarization. The results reveal a rapid effect of both substances on the membrane of $GH_3$/B6 cells. In the case of thyrotropin-releasing hormone, which has both a short-term effect on release of prolactin and a long-term effect on its synthesis, the induced electrical activity may be associated with the stimulation of prolactin production. The physiological implication of 17β-estradiol–induced, calcium-dependent spiking activity remains to be elucidated.*

There is evidence that estrogen steroids can directly regulate the secretory activity of some endocrine (*1*) and neuroendocrine (*2*) cells in the pituitary and the hypothalamus. The effect of estrogen is thought to be mediated through the genome. However, estrogen acts on the brain and pituitary gland with latencies ranging from seconds and minutes to hours and days (*3*), and thus it is unlikely that a single mechanism of action is involved. A short-latency effect of estradiol on electrical activity of hypothalamic neurons (*4*) has already been shown; this effect may be related to a possible regulation of stimulus-secretion coupling at the membrane level (*5*). Furthermore, it has been observed that action potentials are not only a property of neurons but are also detected in endocrine cells, including normal (*6*) or clonal (*7*) pituitary cells. In the present study, intracellular recording was used to demonstrate that estradiol excites pituitary cells, causing a sustained train of action potentials comparable to those induced by thyrotropin-releasing hormone (TRH).

The experiments were performed with $GH_3$/B6 cells, a subclone of the $GH_3$ rat prolactin cell line (*8*), which secrete prolactin (PRL) and are responsive to both TRH (*9*) and estradiol (*10*). The $GH_3$ cells were routinely grown in Ham's F-10 solution supplemented with 15 percent horse serum and 2.5 percent fetal calf serum. Cells were cultured for 5 to 7 days before being used for electrophysiological studies. Experiments were carried out in a temperature-controlled room (26° ± 1°C). Ten minutes before the beginning of a recording session, the culture medium was replaced by a bathing solution of the following composition (in millimoles per liter): NaCl, 142.6; KCl, 5.6; $CaCl_2$, 10; glucose, 5; and Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer, 5 (*p*H 7.4). Stock solutions of 17 β-estradiol (17β-E) and 17α-estradiol (17α-E) (Sigma) were prepared in ethanol and diluted in the bathing solution; the final ethanol concentration was 0.01 percent.

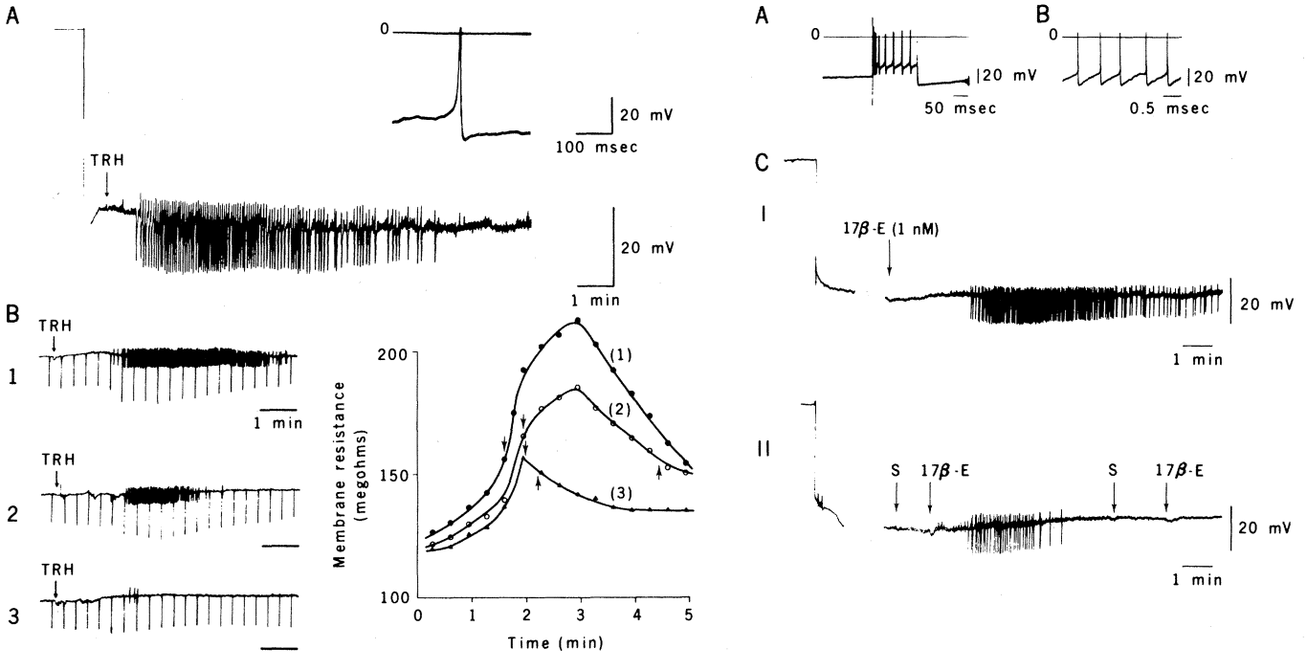


Fig. 1 (left). Effect of TRH on two $GH_3$/B6 cells. (A) Direct injection of TRH (50 n*M*, 2 nl) into the vicinity of the cell membrane (10 μm) elicits sustained spiking activity within 1 minute. The paper recorder could not follow the spikes, which thus appear shorter than they were. The actual shape of the spikes is shown on the oscilloscope trace at the right. Upward deflection indicates a positive potential change. (B) Three successive administrations of TRH (10-minute intervals) evoke successive bursts of activity; however, a desensitization is observed. A measure of the membrane resistance (vertical bars) is obtained by injecting a hyperpolarizing current (0.16 nA) while recording. Bursting activity is preceded by an increase in membrane resistance, the excitability of the cell being directly related to this increase in resistance. The graph shows that the time course of membrane resistance increases following TRH administration at time zero. Arrows indicate the beginning and end of the spiking activity. Fig. 2 (right). Effect of 17β-E (1 n*M*, 2 nl) on the membrane potential of two excitable $GH_3$/B6 cells, I and II. (IA) Excitability of the cell is tested by ejecting a depolarizing current (0.16 nA); (IB) oscilloscope trace showing the actual size of the spike; (IC) injection of 17β-E (1 n*M*, 2 nl) directly onto the membrane of the cell induces a burst of action potentials. (II) Two ejection pipettes are positioned close to the membrane of a $GH_3$/B6 cell, enabling consecutive administrations of 17β-E (1 n*M*, 2 nl) and solvent (2 nl) to be made. Solvent alone does not elicit spiking activity. After a single administration of 17β-E, the cell is no longer excitable and subsequent injections are ineffective.


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The TRH (Beckman) was dissolved in bathing solution. Substances to be tested were delivered via a pneumatic ejection system which allowed rapid administration (0.5 second) of very small volumes (1 to 5 nl) of solution from micropipettes. The tip of the ejecting micropipette was positioned close to the membrane of the cell (*11*). A bridge amplifier was used to pass current through the recording electrode. Glass microelectrodes were pulled from capillary tubes (Clark Electromedical Institute), and those with a d-c resistance of 70 to 100 megohms were used.

Stable intracellular recordings (20 to 30 minutes) were usually obtained, and cells could sometimes be recorded for more than 1 hour. The mean resting membrane potential was 49 ± 8 mV (mean ± standard deviation, $N = 862$). The mean input resistance was 169 ± 58 megohms. Half of the cells appeared electrically excitable and displayed action potentials during a depolarizing intracellular current injection (0.3 to 0.7 nA, 0.5 second). The all-or-none action potential had a positive overshoot and a prominent afterpotential. Some cells displayed a more complex wave form with two distinct rising components, similar to that described in secretory cells of invertebrates (*12*). Twenty-eight percent of the cells were spontaneously active and the firing rate never exceeded 2 Hz. The TRH (25 to 125 n*M*) added to the bathing solution increased the percentage of cells displaying action potentials, which was consistent with the effect of TRH observed with extracellular recording (*6, 7*). Ejection of TRH (50 n*M*, 2 nl) close to the cell evoked a train of action potentials within 1 minute (Fig. 1). This spiking activity was preceded by a progressive increase of the input resistance without any detectable change in the resting membrane polarization. After 1 to 10 minutes the cell stopped firing as the resistance returned to the resting level (Fig. 1). Repeated administration of TRH at 10-minute intervals produced trains of action potentials, although there was a decrease in both the membrane resistance change and the duration of the train (Fig. 1B). Between successive TRH administrations, the level of excitability of the cell did not change, as verified by injection of a depolarizing current. The action potentials were reduced or completely abolished by D 600, a blocker of $Ca^{2+}$ channels. Furthermore the action potentials were only slightly changed in $Na^+$-free medium, although no overshoot was observed. These findings suggest the involvement of $Ca^{2+}$ channels in the spiking activity of pituitary cells. The fact that only 50 percent of the cells were electrically excitable may be due to the asynchronism of mitosis in the cell population.

When 17$\beta$-E was added to the bathing solution (50 pg/ml) the percentage of spontaneously firing cells found during the first hour (49 percent, $N = 25$) was greater than in the nontreated control group (28 percent, $N = 212$). In 31 percent of the excitable cells tested ($N = 58$), 17$\beta$-E ($10^{-10}M$ to $10^{-8}M$) injected close to the cell elicited action potentials within 1 to 2 minutes (Fig. 2). This spiking activity lasted 3 to 30 minutes after application of 17$\beta$-E (*13*) and was reduced by D 600. When an injection of 17$\beta$-E was successful in eliciting spikes, a subsequent injection 3 to 10 minutes later was totally ineffective, although the cell remained excitable by depolarizing current. Like TRH, 17$\beta$-E increased the input membrane resistance before inducing spikes. In contrast, 17$\alpha$-E was almost totally ineffective in producing action potentials; at a much higher concentration ($10^{-6}M$), it elicited a short burst of spikes in only 1 of 23 cells tested. This indicates a considerable degree of stereospecificity for the effects of estrogen on the electrical activity of pituitary cells. Nevertheless, 17$\alpha$-E, when administered 5 minutes before 17$\beta$-E ($N = 18$), prevented the effect of 17$\beta$-E.

Intracellular recordings have confirmed that $GH_3$/B6 rat prolactin cells display spontaneous and TRH-induced electrical activity (*7*). The action potentials appear to be calcium-dependent, since they were inhibited or partly suppressed by the calcium blocker D 600 and persisted in $Na^+$-free solution. This is consistent with the recent demonstration of the role of $Ca^{2+}$ in TRH-stimulated release of PRL by $GH_3$ cells (*14*) and is in agreement with the concept of a stimulus-secretion coupling mechanism (*15*). In addition, the effect of TRH on the spiking activity of $GH_3$/B6 cells and the calcium dependence are similar to effects previously observed with extracellular recording in normal pituitary cell cultures (*6*). The clonal cell line $GH_3$/B6 could therefore be used as a model system in which to study electrophysiological properties of adenohypophyseal cells. In contrast to what was expected, there appeared to be no change in membrane polarization preceding the spiking activity induced by TRH. However, an increase in the membrane resistance was found to be necessary for the initiation of a train of spikes. Such a phenomenon has also been reported after injection of hypothalamic extracts in vivo, where again an increase in membrane resistance was observed with no change in the membrane potential (*16*).

Our results also reveal a rapid effect of 17$\beta$-E on the membrane of $GH_3$/B6 cells. The electrical activity elicited by 17$\beta$-E was similar in time course and $Ca^{2+}$ dependence to that induced by TRH. In contrast to the progressive desensitization to TRH, total desensitization was observed after the first injection of 17$\beta$-E. The rapid and specific effect of 17$\beta$-E on membrane properties implies recognition sites for the steroid at the membrane surface and probably reflects conformational changes in membrane components. Recent evidence for a membrane site of action of estrogen has been reported for uterine cells (*17, 18*).

In the case of TRH, which exerts both a short-term effect on PRL release and a long-term effect on PRL synthesis (*19, 20*), the TRH-induced electrical activity may be associated with the stimulation of PRL release (*7, 14*). 17$\beta$-Estradiol has been shown to have a long-term effect on PRL synthesis (*10*). Nevertheless, the possibility that PRL release is stimulated within minutes after 17$\beta$-E injection, which would be consistent with the $Ca^{2+}$-dependent electrical effect, has not yet been investigated. It is also possible that $Ca^{2+}$ is required to initiate the long-term effect of 17$\beta$-E on PRL synthesis.

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13. Surprisingly, this effect was more marked and was observed in 50 percent of the total population when TRH ($50 \times 10^{-6}$ to $125 \times 10^{-6} M$) had been added to the medium 4 to 6 hours before. At present, we are unable to explain the fact that TRH apparently enhances the sensitivity of $GH_3$ cells to the action of 17$\beta$-E.
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## DNA Polymerase with Characteristics of Reverse Transcriptase Purified from Human Milk

Abstract. *A DNA polymerase purified from a particulate fraction of human milk has biochemical and biophysical properties similar to those of viral reverse transcriptases. This enzyme is immunologically distinct from cellular DNA polymerases obtained from a variety of human sources.*

A number of studies (*1, 2*) have shown that some human milk samples contain an RNA-dependent DNA nucleotidyl transferase activity (reverse transcriptase). This enzyme has been isolated from a variety of human tissues and malignant cells (*3*). However, purified reverse transcriptase has been immunologically characterized only three times: from human leukemic cells (*4*), preleukemic spleen (*5*), and breast cancer cells (*6*). The leukemic and preleukemic enzymes were immunologically related to the reverse transcriptase of the gibbon ape leukemia virus and the simian sarcoma virus (*4, 5*). The DNA polymerase isolated from human breast cancer was immunologically related to the reverse transcriptase of the Mason Pfizer monkey virus (*6*).

We report here the purification of a DNA polymerase from high-density particles obtained from human milk. This enzyme resembles the reverse transcriptase of RNA tumor viruses in its primer template specificities and cation requirement. The purified enzyme was devoid of terminal transferase activity and was not immunologically related to human DNA polymerase $\alpha$, $\beta$, or $\gamma$.

High-density particles ($\rho > 1.20$) were obtained from defatted human milk (*2, 7–9*) and the DNA polymerase activity was purified from these particles by sequential chromatography on DEAE-52 cellulose (*8*) and polyribocytidylic acid agarose [poly(C) agarose]. Figure 1, A and B, shows elution profiles of the human milk DNA polymerase from DEAE-52 cellulose and from poly(C) agarose. The enzyme eluted as a single peak of activity from the poly(C) agarose column at a concentration of 0.22*M* KCl.

The molecular weight of the DNA polymerase was determined by three methods: (i) SDS-polyacrylamide gel electrophoresis, (ii) Sephadex G-200 chromatography, and (iii) velocity sedimentation analysis. Electrophoresis was performed on 10 percent polyacrylamide gels in the presence of 0.1 percent SDS (*10*). Molecular weights of the separated polypeptides were determined by using phosphorylase A (94,000), bovine serum albumin (68,000), and ovalbumin (43,000) as molecular weight markers. The poly(C) agarose peak material, with a 200-fold purification over the high-density particles, contained a major polypeptide band corresponding to a molecular weight of 70,000. Molecular sieving of the material by Sephadex G-200 in the presence of 0.5*M* KCl also indicated that the human milk DNA polymerase had a molecular weight of 70,000 (Fig. 1C). The DNA polymerase was sedimented through a linear glycerol gradient (10 to 30 percent by volume) and the enzyme-active fractions were located by assaying with the primer template $(dG)_{12-18} \cdot (C)_n$. The enzyme activity sedimented between 5*S* and 5.5*S*, slightly faster than the bovine serum albumin marker (data not shown).

Cellular DNA polymerases can be distinguished from one another and from viral reverse transcriptases by their synthetic primer-template specificities (*11*). In general, the viral reverse transcriptases show a preference for $(dT)_{12-18} \cdot (A)_n$ and not $(dT)_{12-18} \cdot (dA)_n$ (*12*). They will also use $(dG)_{12-18} \cdot (C)_n$ and $(dG)_{12-18} \cdot (Cm)_n$ (*13*) as templates for the synthesis of poly(dG). The cellular DNA polymerase, specifically polymerase $\gamma$, will inefficiently transcribe $(dG)_{12-18} \cdot (C)_n$ at low salt concentrations, that is, 0.05*M* $KPO_4$ or no added KCl (*13, 14*), and will not utilize poly(2′-*O*-methylcytidylate) · oligodeoxyguanylate for the synthesis of poly(dG) (*14*).

The response of the human milk DNA polymerase to synthetic primer templates appears in Table 1. The enzyme can synthesize poly(dT) in the presence of $(dT)_{12-18} \cdot (A)_n$ and $Mg^{2+}$ (10 m*M*). It will also utilize the templates $(dG)_{12-18} \cdot (C)_n$ and $(dG)_{12-18} \cdot (Cm)_n$

Table 1. Enzymatic activity of the human milk DNA polymerase with various synthetic polynucleotides being used as templates. Assay mixtures containing 10 $\mu$l of enzyme were initiated by adding 40 $\mu$l of a mixture that gave a final concentration of 50 m*M* tris-HCl (*p*H 8.0); 60 m*M* KCl; 1 m*M* DTT; either 10 m*M* $MgCl_2$ or 0.5 m*M* $MnCl_2$, 7.6 $\mu M$ [$^3$H]TTP (2000 to 4000 counts per minute per picomole) or 7.6 $\mu M$ [$^3$H]dGTP (2000 to 4000 counts per minute per picomole); and bovine serum albumin (0.5 mg/ml). All reactions were performed at 37°C for 30 minutes. Acid-insoluble precipitates were collected on filters and the radioactivity was counted by means of a liquid scintillation counting system.

| Template | [$^3$H]dNTP | Divalent cation | [$^3$H]dNMP, polymerized (pmole) |
|---|---|---|---|
| $(A)_n \cdot (dT)_{12-18}$ | TTP | $Mn^{2+}$ | 0.16 |
| $(A)_n \cdot (dT)_{12-18}$ | TTP | $Mg^{2+}$ | 0.70 |
| $(dA)_n \cdot (dT)_{12-18}$ | TTP | $Mn^{2+}$ | 0.10 |
| $(dA)_n \cdot (dT)_{12-18}$ | TTP | $Mg^{2+}$ | 0.08 |
| $(C)_n \cdot (dG)_{12-18}$ | dGTP | $Mn^{2+}$ | 0.05 |
| $(C)_n \cdot (dG)_{12-18}$ | dGTP | $Mg^{2+}$ | 0.50 |
| $(Cm)_n \cdot (dG)_{12-18}$ | dGTP | $Mn^{2+}$ | 0.25 |
| $(Cm)_n \cdot (dG)_{12-18}$ | dGTP | $Mg^{2+}$ | 0.50 |
| | *Primer alone* | | |
| $(dT)_{12-18}$ | TTP | $Mg^{2+*}$ | < 0.01 |
| $(dT)_{12-18}$ | dGTP | $Mg^{2+*}$ | < 0.01 |
| $(dG)_{12-18}$ | TTP | $Mg^{2+*}$ | < 0.01 |
| $(dG)_{12-18}$ | dGTP | $Mg^{2+*}$ | < 0.01 |

*Same results with $Mn^{2+}$


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