Membrane potential changes caused by thyrotropin-releasing hormone in the clonal GH₃ cell and their relationship to secretion of pituitary hormone

(action potential/calcium/cobalt/prolactin/growth hormone)

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ABSTRACT Effects of thyrotropin-releasing hormone (TRH; thyroliberin) on membrane electrical properties were studied in the clonal rat anterior pituitary cell (GH₃) by continuous recording of the intracellular potential. Application of TRH, which stimulates the release of prolactin and growth hormone from the GH₃ cell, elicited a transient hyperpolarization of the cell membrane followed by an enhancement of the generation of action potentials for an extended period in the majority of cells tested. The transient hyperpolarization was due to an increase of the membrane conductance to K⁺. The enhancement of the spike generation was not due to membrane depolarization. The input resistance of the cell membrane was found to be increased during the facilitation. Thus the mechanism of the facilitatory action of TRH is different from the mechanisms of conventional excitatory neurotransmitters. TRH enhances the spike generation, thus promoting Ca²⁺ entry from extra- to intracellular space (the action potential of the GH₃ cell has a Ca²⁺ component) and stimulating the release of hormones. This notion is supported by the observation that cobalt ions, which block the calcium spike in these cells, completely abolished the stimulatory effect of TRH on the release of prolactin and growth hormone.

Release of anterior pituitary hormones is regulated by hypothalamic-hypophyseotropic hormones, which in three cases have been chemically identified as peptides (1, 2). However, the mechanism by which these peptides act on target pituitary cells remains to be elucidated. The GH₃ strain of rat pituitary cells secretes prolactin and growth hormone, and the acute release of both these hormones is stimulated by thyrotropin-releasing hormone (TRH; thyroliberin) (3). These clonal cells have been used as a suitable model for studying the action mechanism of TRH (4, 5). Electrophysiological experiments have shown that the GH₃ cell is capable of generating action potentials with a Ca²⁺ component (6-8). Furthermore, by using an extracellular recording technique it has been shown that the frequency of action potentials is significantly increased in the presence of a small amount of TRH in the external medium. The above observations suggest that TRH may exert its stimulating effect on the hormone release from the GH₃ cell by modulating the frequency of action potentials (6). In order to determine the mechanism of enhancement of the spike generation, it is necessary to study the effects of TRH on membrane electrical properties of the GH₃ cell. In the present communication we report TRH-induced changes of the membrane properties revealed by continuous recordings of the intracellular potential. Further, we demonstrate that cobalt ions abolish both calcium action potentials and the TRH-induced release of prolactin and growth hormone in the GH₃ cell, which suggests

that calcium ions entering the cell during the occurrence of action potentials are essential for the stimulatory action of TRH on the release of the hormones.

During preparation of the present communication, a short note by Dufy *et al.* (9) appeared that showed that TRH causes a burst of action potentials without any detectable change in the resting membrane potential. We confirm the results of Dufy *et al.* on the enhanced action potential generation by TRH. In addition, we find a transient hyperpolarization preceding the facilitation in the majority of cells.

MATERIALS AND METHODS

Cells and Solutions. The clonal cell strain GH₃ was obtained commercially from the American Type Culture Collection through Dainihon-Seiyaku (Osaka, Japan). The cells were grown in plastic dishes containing Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Most experiments were performed in a standard solution that had the same composition as Ham's F-10 medium except that: (i) the concentrations of Ca²⁺ and K⁺ were adjusted to 2.4 and 5.0 mM, respectively, (ii) NaHCO₃ was replaced with NaCl and the solutions were buffered by 10 mM Hepes/NaOH or Tris-HCl to pH 7.4. Na⁺-free solution was made by replacement of Na⁺ with isomolar choline⁺. Some experiments were done in normal saline, which had the following composition (mM): NaCl 150, KCl 5, CaCl₂ 2.4, MgCl₂ 1.3, glucose 10, and Hepes/NaOH buffer 10, at pH 7.4. The experimental results were essentially similar for cells in this saline and standard solution. Cl⁻-free solution was made by substitution of propionate for the Cl⁻ in this saline.

Electrophysiology. Details of the electrophysiological methods for recording intracellular potentials from GH₃ cells have been described elsewhere (8).

Test solutions of 5 μM TRH (dissolved in the bathing solution), 100 mM Co²⁺, and 50-500 mM K⁺ were applied to the cell from a delivery pipette. The pipette was prepared by using a microforge, and the inner tip diameter was 3 μm throughout the present experiments. The shaft of the solution-filled pipette was connected to a metal syringe with a screw-operated plunger by a polyethylene tube filled with olive oil. After a stable recording had been obtained, the pipette was positioned about 30 μm from the cell. The test solution was then ejected by applying pressure. In some experiments, a delivery pipette with a coarse tip (tip diameter > 5 μm) was used for application of TRH without pressure ejection. When this pipette was brought near the cell, a typical response to TRH was also observed, presumably due to leakage of TRH from the pipette.

Abbreviation: TRH, thyrotropin-releasing hormone (thyroliberin).

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The electrophysiological experiments were carried out at 31–36°C. It was previously reported that spontaneous action potentials occurred in a small number of cells in the normal saline at room temperature (23–25°C) (8). In the present series of experiments, spontaneous firing was observed in most cells in both standard and Na^+ -free solution when the resting potential was less negative than -44 mV. The higher temperature could be more favorable for initiation of spontaneous action potentials in the GH₃ cell.

Assay of Prolactin and Growth Hormone. Prolactin and growth hormone released from the GH₃ cell into the incubating media were measured by the following procedure. A cell suspension of 1×10^5 cells per ml was prepared, and 2.5-ml aliquots of the suspension were placed in Falcon dishes (35 $\times$ 10 mm). The experiment was conducted 7 days after plating. The cells were washed three times with normal saline before the experiment started, and they were then incubated in 2 ml of the standard solution for 1 hr at 37°C. The amounts of prolactin and growth hormone secreted into the media were determined by radioimmunoassay for rat prolactin and growth hormone, utilizing reagents provided by the National Institute of Arthritis, Metabolism and Digestive Diseases (NIAMDD). The cell protein was measured by the method of Lowry *et al.* (10).

Data are expressed as mean $\pm$ SEM. Statistical evaluation of the results was done with Student's *t* test.

RESULTS

Effects of TRH on the membrane electrical properties

The responses of the cell membrane to TRH were examined in 70 cells in standard solution. They could be classified into three types: (i) In 38 cells, TRH produced a long-lasting enhancement of spike generation preceded by a transient membrane hyperpolarization, as shown in Fig. 1A (type 1 response). (ii) In 7 cells, TRH enhanced spike generation unaccompanied by the membrane hyperpolarization, as shown in Fig. 1B (type 2 response). (iii) In 8 cells, TRH produced only the transient membrane hyperpolarization without the following facilitation (type 3 response). TRH had no influence on the electrical properties of the cell membrane in the remaining 17 cells. The following description will be mainly concerned with type 1 response, which occurred in the majority of cells (see *Discussion*). The transient hyperpolarization of type 1 response lasted for 5–15 sec (9.8 ± 0.7 sec, $n = 38$). In order to understand the nature of this hyperpolarization, the membrane conductance was measured by pulsing current of constant intensity through the recording electrode. As shown in Fig. 1D, the conductance increased during the hyperpolarizing phase. When the membrane potential was maintained at a level more negative than -70 mV by dc hyperpolarizing current, the polarity of this transient response was reversed. However, the response was still associated with an increase in the membrane conductance (Fig. 1E). The reversal potential was estimated by the method illustrated in Fig. 1F. When the peak level of the electrotonic potential caused by the hyperpolarizing current pulse exceeded about -70 mV, the envelope of the peak level of the electrotonic potential was shifted to a depolarizing direction by TRH application (*inset* of Fig. 1F). The amplitude of the hyperpolarization due to TRH in the steady state (*a* in *inset* of Fig. 1F) and that of the depolarizing shift of the peak level of the electrotonic potential (*b* in *inset* of Fig. 1F) were plotted against the resting potential and the polarized potential level, respectively. The reversal potential was taken as the membrane potential at which the straight line connecting these two points crossed the abscissa. Its mean value was -66.5 ± 1.1 mV ($n = 7$). The above observation indicates that the TRH-induced hyperpolarization may

FIG. 1. Effects of TRH on the membrane potential. TRH was applied from delivery pipettes filled with $5 \mu\text{M}$ TRH by pressure at the moments indicated by ■s, except for C. (Aa) Pen recorder trace showing the whole time course of the response to TRH found in the majority of cells. The resting potential was -42 mV. (Ab) Oscilloscope trace of the underlined part in record Aa. (B) Response to TRH that occurred in a small number of cells. Only the enhancement of the spike frequency was induced, unaccompanied by the hyperpolarization. The resting potential was -38 mV. The traces are interrupted for 2 sec in the middle in B, C, and D. (C) TRH was applied from a delivery pipette with a coarse tip (inner diameter $>5 \mu\text{m}$) without applying pressure. The pipette was filled with 0.1 mM TRH and positioned close to the cell during the period indicated by the thick line above the potential trace. Note the transient hyperpolarizing response despite the continuous leakage of TRH from the pipette. The spike frequency was increased from 1.3 to 2.1 Hz after the hyperpolarization. The resting potential was -41 mV. (D) Changes in the membrane conductance. As shown in the lower trace, constant current pulses of 300-msec duration were passed through the recording electrode at 1 Hz. (E) Response to TRH with the membrane potential maintained at a level more negative than -70 mV by dc hyperpolarizing current. (F) Estimation of the reversal potential of the TRH-induced hyperpolarization. An example of potential traces used for this purpose is illustrated in the *Inset*. The dotted line shows the envelope of the peak of the electrotonic potential. The arrow in the graph shows the level at which the reversal is expected to occur (see text). The calibrations of 2 sec and 20 mV below record E apply to C, D, E, and the *Inset* of F. The current calibration of 10^{-10} A applies to the lower traces of D and E.

be due to an increase in the membrane permeability to K^+ , Cl^- , or both. However, permeability change to Cl^- does not contribute to generation of this response, because the hyperpolarizing response with a similar increase in the membrane conductance was also found in Cl^- -free solution. It can therefore be concluded that the transient hyperpolarization is due to an increase in the membrane permeability to K^+ .

The transient hyperpolarization was followed by a phase in which the spike initiation was augmented (Fig. 1A, C, and D). Repetitive firing was initiated in quiescent cells in which the resting potential was less negative than -44 mV, and the firing frequency was increased in spontaneously active cells (Fig. 1A and C). This facilitation usually lasted more than 50 sec. During this facilitatory phase the input resistance of the cell membrane increased (Fig. 1D). The mean increase was $31 \pm$

5% ($n = 16$). The resting potential level occasionally depolarized by 1–5 mV in this phase (not shown in the figure). However, facilitation occurred without detectable depolarization in most cases (see Fig. 1 A and C), which precludes that the depolarization is a primary cause of the enhancement of spike generation.

The sequence of responses to TRH, a transient membrane hyperpolarization followed by facilitation of the spike generation, might be interpreted as follows: The effect of TRH is exclusively to increase the K^+ conductance of the cell membrane, and the membrane thus hyperpolarizes whenever the cell is surrounded by TRH above a critical concentration. The enhancement of spike generation could then be an off-response to the inhibitory hyperpolarization. However, this notion is incompatible with the observation shown in Fig. 1C. TRH was here applied for prolonged periods from a coarse delivery pipette, but the hyperpolarizing response was short-lasting even under such conditions. Furthermore, the long-lasting facilitation could not be produced as an off-response when the hyperpolarizing response to TRH was mimicked by passing current through the recording electrode.

The TRH-induced changes, both hyperpolarization and after-facilitation, also occurred in Na^+ -free solution.

Co^{2+} blocks the calcium spike and TRH-stimulated hormone release

Co^{2+} has a blocking effect on the current of the calcium channel in various tissues producing calcium spikes (11). It has been shown that the calcium spike in the GH_3 cell is abolished by 2–4 mM Co^{2+} (8). In this experiment, the blocking effect of Co^{2+} on the calcium spike recorded in Na^+ -free solution was tested in a single cell by applying Co^{2+} from a delivery pipette containing 100 mM $CoCl_2$. Calcium-dependent action potentials occurred spontaneously when the resting potential was less negative than -44 mV. These calcium spikes were abolished by application of Co^{2+} (Fig. 2A). The calcium spike elicited by passing a depolarizing current pulse was also blocked reversibly by the same procedure (Fig. 2B).

If the calcium spike is involved in the stimulatory action of TRH on the hormone release in the GH_3 cell, the effect of TRH will be abolished under conditions in which the occurrence of the calcium spike is blocked. This expectation was corroborated by the following experimental results. Fig. 3 shows that the rate of release of both prolactin and growth hormone increased significantly ($P < 0.01$ for each comparison) when 100 nM TRH was added to the standard solution. The stimulatory effect of

FIG. 2. Reversible blocking effects of Co^{2+} on the calcium action potentials in Na^+ -free solution. Co^{2+} was delivered from pipettes filled with 100 mM $CoCl_2$ solution. (A) Spontaneous action potentials were reversibly abolished by application of Co^{2+} at the moment shown by ■. The trace is interrupted for 2 sec in the middle. The resting potential was -42 mV. (Ba) Action potential elicited by a depolarizing current pulse in Na^+ -free solution. (Bb) The action potential was abolished after application of Co^{2+} . (Bc) The action potential reappeared 1.3 sec after application of Co^{2+} . The resting potential was -52 mV.

FIG. 3. Blocking effects of Co^{2+} on the TRH-stimulated release of prolactin (A) and growth hormone (B) from GH_3 cells. The empty bars represent the mean quantities $\pm$ SEM of prolactin or growth hormone secreted during 1 hr into a standard medium, and the filled bars are for a medium containing 100 nM TRH. The sample number in each case was three. The data are expressed as ng of rat prolactin (NIAMDD rat prolactin RP-1) or ng of rat growth hormone (NIAMDD rat GH-RP-1) per mg of cell protein per hr.

TRH was inhibited in the presence of 2 mM Co^{2+} (Fig. 3). A similar effect of Co^{2+} has also been reported for the GH_4C_1 strain of rat pituitary cells (12). It could be argued that unphysiological substances, such as cobalt ions, probably interfere with the binding of TRH to the receptor on the cell membrane, thereby inhibiting the stimulatory action of TRH on the hormone secretion. In the presence of 2–4 mM Co^{2+} , however, TRH induced the transient hyperpolarization associated with an increase in the membrane conductance and the following increase in the membrane input resistance. This finding indicates that the action of TRH on the cell membrane is not affected by the presence of Co^{2+} . From these observations it is most likely that TRH increases the calcium entry (by enhancing spike generation) into the cell through the voltage-dependent calcium channels and thereby promotes the hormone release from the GH_3 cells.

DISCUSSION

Application of TRH produced a membrane hyperpolarization followed by enhancement of the spike generation in the majority of cells. Different types of response, however, occurred in a minority of cells: one is facilitation without the transient hyperpolarization (type 2 response), and the other is a transient hyperpolarization not followed by the facilitation (type 3 response). As described above, the TRH-induced hyperpolarization of the type 1 response was transient, even if TRH was applied for a prolonged period (Fig. 1C). It also tended to be gradually reduced by repeated administration of TRH; and in some extreme cases, even the second application of TRH immediately enhanced the spike generation without eliciting the hyperpolarization (unpublished data). Such an evanescent property of the TRH-induced hyperpolarization may lead to the suggestion that a single GH_3 cell could show either type 1 or type 2 response according to some unknown changes in the cell condition. In the type 3 response, the failure of occurrence of the after-facilitation was due to a gradual hyperpolarization of the cell membrane that had started before the membrane potential was restored to the original level from the TRH-induced hyperpolarization. This hyperpolarization usually reached a steady level more negative than -60 mV within several minutes. During this hyperpolarization, the membrane

conductance markedly increased and the ability of the cell membrane to generate action potentials was gradually lost and was never regained. This irreversible change was, most likely, not a TRH-induced one, because it was frequently observed before TRH had been applied. Therefore, we interpret that the after-facilitation of spike generation was prevented by the irreversible hyperpolarizing change in the cells showing the type 3 response.

The latency for the TRH-induced response was shorter in the present experiments than that reported by the previous authors (9). The transient hyperpolarizing response usually occurred within several seconds after TRH administration. Because the hyperpolarizing response lasted for 5–15 sec, the facilitation started within 20 sec in most cases. This discrepancy could be due to differences in the concentration of TRH applied. In our experiments, the delivery pipette was filled with 5 μ M TRH. The concentration of TRH at the cell surface would be much lower than this value even at its peak. This statement is based on the data obtained from the measurement of the peak amplitude of the membrane depolarization induced by high K^+ concentrations ejected from delivery pipettes containing 50–500 mM KCl solution. The peak K^+ concentration around the cell concerned was estimated to be diluted to 1/30th to 1/40th of that in the delivery pipette under the assumption that the membrane depolarizes according to Nernst equation. However, the concentration of TRH at the cell surface could still be higher in the present experiments than in the previous authors', in which 2 nl of 50 nM TRH solution was applied from micropipettes. Such difference in the concentration of TRH could also cause the discrepancy in the two results with regard to the presence of TRH-induced hyperpolarization.

The TRH-induced hyperpolarization was due to an increase in the K^+ conductance. Because intracellular Ca^{2+} injection is known to increase K^+ conductance (13), it is tempting to speculate that TRH increases the K^+ conductance by mobilizing the intracellular calcium stored in the cell organelles or the membrane. If so, the transient hyperpolarizing response could be closely related to the TRH-stimulated hormone release. However, this notion is not consistent with the observation that the hyperpolarizing response to TRH also occurred in solution containing 2–4 mM Co^{2+} in which the TRH-mediated hormone release was completely abolished.

The facilitatory action of TRH on the GH_3 cell is different from that of conventional excitatory transmitters at synapses of excitable tissues in the following respects: (i) The action was long-lasting. (ii) The enhancement of the spike initiation was not due to a sustained membrane depolarization. (iii) The response also occurred in Na^+ -free solution, which indicates that a change in the Na^+ conductance is not indispensable for initiation of this facilitation. We confirmed the results of Dufy *et al.* (9) that the input resistance of the cell membrane increases during the facilitatory phase. This increase may play a role in enhancing the spike generation by decreasing an outward leak current. However, we cannot exclude the possibility that other factors also contribute to this electrical event. It has been reported that addition of TRH to the bathing solution causes an increase in the number of cells that fire spontaneously and that more than half of those cells display prolonged action potentials (14). These observations may lead to a suggestion that TRH facilitates initiation of the regenerative process by affecting the time- and voltage-dependent Ca^{2+} , K^+ , or both channels. In

this context it is intriguing that 4-aminopyridine, which is known to block the K^+ channel (15), induced a repetitive firing as well as prolonged action potentials in GH_3 cells (14).

It has recently been demonstrated that the anterior pituitary cells in the rat and fish generate action potentials due to an increase in the membrane permeability to both Na^+ and Ca^{2+} (16–18). TRH has been shown to enhance the spike initiation in about 10% of the pituitary cells in primary culture obtained from rats (16). Further, it is known that the action of the hypothalamic releasing hormone is calcium-dependent, and that the release of the pituitary hormones is enhanced by incubating the pituitary gland in high K^+ solution in the presence of Ca^{2+} (19–21). Therefore, it is possible that the action of the releasing hormone is exerted by affecting the spike generation in the anterior pituitary cells *in situ* as well as in the GH_3 cells.

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