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# AUGMENTATION OF TRANSIENT LOW-THRESHOLD $Ca^{2+}$ CURRENT INDUCED BY GTP-BINDING PROTEIN SIGNAL TRANSDUCTION SYSTEM IN $GH_3$ PITUITARY CELLS

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**SUMMARY** We characterized the effects of thyrotropin-releasing hormone (TRH; 500 nM) and guanosine 5'-0-3-thiotriphosphate (GTP$\gamma$S; 50 $\mu$M) on two types of $Ca^{2+}$ currents in pituitary-hormone-secretory $GH_3$ cells and were surprised to find marked increases in transient, low-threshold $Ca^{2+}$ currents (T currents) induced by extracellularly applied TRH or intracellularly applied GTP$\gamma$S. The effect of TRH was blocked by intracellularly applied guanosine 5'-0-2-thiodiphosphate (GDP$\beta$S; 100 $\mu$M). The increase in the T current was found to be accompanied by a decrease in long-lasting, high-threshold $Ca^{2+}$ current (L-current), in response to both TRH or GTP$\gamma$S. These indicate that the enhancement of $Ca^{2+}$ influx by TRH (500 nM) is largely conferred by T currents in $GH_3$ cells. A reduced concentration of TRH (5 nM) still markedly increased the T current, but failed to decrease the L current. These data suggest that the augmentation of the T currents as well as depression of the L currents by TRH (500 nM), through the activation of a GTP-binding protein, may constitute an important regulatory mechanism of sustained pituitary hormone secretion in $GH_3$ cells. 

The modulation of $Ca^{2+}$ channels by the neuropeptide thyrotropin-releasing hormone (TRH), may be an important mechanism of regulating the sustained increase in cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$), which results in sustained hormone secretion (1-3), as well as inhibition of voltage-dependent $K^+$ currents by TRH in $GH_3$ cells (4,5).

Previous whole-cell studies in $GH_3$ cells demonstrated the presence of transient low-threshold (T-type)- and long-lasting high-threshold (L-type)-, $Ca^{2+}$ channels (6-8), but not of the transient high-threshold (N)-type. T-

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Abbreviations used; GTP$\gamma$S, guanosine 5'-0-3-thiotriphosphate; GTP, guanosine 5'-triphosphate; GDP$\beta$S, guanosine 5'-0-2-thiodiphosphate; $[Ca^{2+}]_i$, cytosolic free $Ca^{2+}$ concentration; T current, transient low-threshold $Ca^{2+}$ current; L current, long-lasting high-threshold $Ca^{2+}$ current.

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and L- channels function very differently in the conversion of action potentials to intracellular calcium transients, as evidenced by McCobb and Beam (9). The individual roles of the two distinct types of voltage-dependent $Ca^{2+}$ channel in the specialized hormonal function in $GH_3$ cells remain to be determined.

We have recently suggested that the sustained elevation of $[Ca^{2+}]_i$ triggered by TRH in $GH_3$ cells might be due to repetitive openings of T-type $Ca^{2+}$ channels causing a series of transient $Ca^{2+}$ influx largely through T-channels (10), as a result of the enhancement of action potential firing over a prolonged period induced by TRH effect in $GH_3$ cells (4, 11-14; for a review, see 15). Also, TRH inhibited L-type $Ca^{2+}$ current has been clearly demonstrated by Levitan and Kramer (8) and Kramer et al. (16), using the whole-cell perforated patch clamp technique. This result further suggests that $Ca^{2+}$ influx through T-type $Ca^{2+}$ channels is responsible for the sustained increase of $[Ca^{2+}]_i$.

In this whole-cell voltage-clamp study, we concentrated on examining whether TRH modulates the T-type and L-type currents in $GH_3$ cells, through the activation of a G protein, which may regulate the frequency of $Ca^{2+}$ action potentials, thereby controlling the level of the sustained $[Ca^{2+}]_i$ in $GH_3$ cells.

## MATERIALS AND METHODS

Cell culture A cloned line of rat pituitary tumor cells, $GH_3$ cells, was purchased from American Type Culture Collection (Rockville, MD) through Dainihon-Seiyaku (Oosaka, Japan), and the cells were maintained in Ham's F-10 medium supplemented with 15% horse serum, 2.5% fetal bovine serum under 5% $CO_2$/ air at 37 °C (17). The medium contained 50 units/ ml penicillin and 50 $\mu$g/ ml streptomycin. Cells were plated onto glass coverslips (15 mm diameter) and electrophysiological experiments were performed 2-7 days later.

Physiological recording The whole-cell patch-clamp technique (18) was used to examine cells in a bath perfused (1 ml/min) at room temperature. The patch electrode resistance ranged from 3 to 8 MΩ when filled with internal solutions. A model S-3666 patch clamp whole cell clamp amplifier (Nihon Kohden, Tokyo) with a type P-10G probe (feedback resistor: 10 GΩ) was used. Voltage pulses and currents were sampled online at 200 $\mu$sec intervals with a computer (VM-2, NEC, Japan). Whole cell current measured under voltage clamp conditions was sampled after filtered (+3 db at 3 kHz). The stimulation rate was 0.5 Hz. Linear leak and capacitance currents were subtracted digitally.

$Ca^{2+}$ currents were recorded with a pipete filled with an intracellular solution containing 145 mM Cs aspartate, 5 mM EGTA, 2 mM Mg-ATP, 10 mM HEPES (pH 7.4); cells were continuously perfused with $Ca^{2+}$-TEA saline consisting of 2 mM $CaCl_2$, 138 mM TEA-Cl, 2 mM $MgCl_2$, 20 mM HEPES (pH 7.3 was adjusted by NaOH). Tetrodotoxin (TTX) at a concentration of 500 nM was added to suppress currents through $Na^+$ channels. TRH (Sigma) in distilled water was dissolved in the perfusing saline. Nonhydrolyzable GTP analog, guanosine 5'-0-3-thiotriphosphate (GTP$\gamma$S; Boehringer-Mannheim), an activator of G protein (19), was added to the patch pipet solution at a concentration of 20 $\mu$M in Figs. 1 and 3. In Fig. 4B, GTP$\gamma$S was added to the patch pipete solution at 50 $\mu$M. In some experiments, hydrolyzable guanosine 5'-triphosphate (GTP, Boehringer-Mannheim) was added to the patch pipete solution at 20 $\mu$M. Nonhydrolyzable

GDP analog, guanosine 5'-0-3-thiotriphosphate (GDP$\beta$S; Boehringer-Mannheim), an inactivater of G protein (19), was added to the patch pipet solution at a concentration of 100 $\mu$M in Fig. 2. Solution changes in the chamber were completed within 3 min. Other chemicals were from Wako Pure Chem. (Oosaka, Japan).

## RESULTS

### T- and L-channels in $GH_3$ cells.

Figure 1A (control traces) shows that, from a holding potential of -80 mV, depolarizing test pulses to between -50 and -30 mV (potential indicated above each traces) induced T-type currents showing rapid inactivation with maintained depolarization, and test pulses at -20 and -10 mV produced additionally L-type currents showing very slow inactivation during maintained depolarization. The amplitude of L-current was measured at the end of 160 msec test pulses (for an example, see the arrow heads in Fig. 1A), at which time the T-current components were presumably fully inactivated. In Fig. 1, the associated current-voltage relations depict the peak (= T + L) current (B), L current (C) and T current (D), measured in control solution (open circles). To isolate the amplitude of T current (D), the amplitude of L current (C) was subtracted from the amplitude of peak current (B). In the experiments of Figs. 1 and 3, GTP$\gamma$S was added to the patch pipet solution at a concentration of 20 $\mu$M, although we could not know actual concentrations of GTP$\gamma$S rendered within cells.

### Effects of TRH (500 nM) on T- and L-currents.

Figure 1A (TRH traces) shows that TRH (500 nM) dramatically increased the T current amplitude in $GH_3$ cells. A significant increase of T current produced by TRH was seen over test potential range of -50 to -10 mV, with no appreciable change in the threshold potential of about -60 mV (Fig. 1D; solid circles). In contrast to the increase in the amplitude of T current, Figure 1A (TRH traces) illustrates that TRH (500 nM) dramatically decreased the amplitude of L current, as shown at test pulses of -20 and -10 mV. The decrease in the amplitude of L current by TRH was seen at test potentials exceeding -20 mV (Fig. 1C; solid circles). Figure 1A (Recovery traces) shows that both this decrease in L current and the increase in T current were appreciably reversed when TRH was washed out (see also open triangles in Figs. 1C and 1D), indicating that the decrease of L current was not caused by "run down" at least to the extent of recovery, but rather by a depressive effect of TRH.

Superimposed $Ca^{2+}$ current traces in Fig. 2 were measured in the cell including GDP analog GDP$\beta$S (100 $\mu$M) introduced with a patch pipet, an inactivator of G protein (19). In Fig. 2B, the addition of TRH (500 nM) produced no significant changes in the T currents (compare B with A), although some

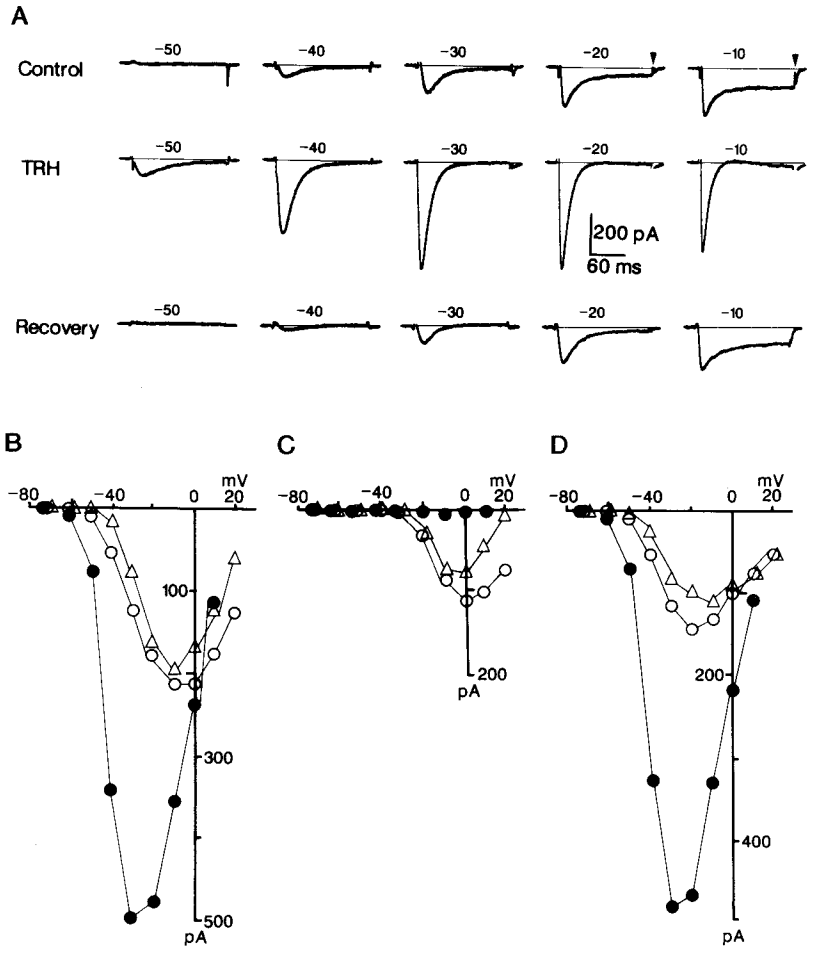


Fig. 1. TRH (500 nM) increased T-type $Ca^{2+}$ channel current, but reduced L-type $Ca^{2+}$ channel current. Currents were recorded under whole-cell voltage-clamp in $GH_3$ cells. In (A), $Ca^{2+}$ current traces were obtained just before (Control traces), during application of 500 nM TRH (TRH traces) and following recovery of 5 min (Recovery traces), to the same $GH_3$ cell. From a holding potential of -80 mV, depolarizing pulses (160 msec duration) to between -50 and -30 mV, the potential indicated above each traces, induced T-type current, and test pulses at -20 and -10 mV in (A) and -20 and +20 mV in (B) produced L-type current, together with T-type current. The amplitude of L-type current was measured at the end of 160 msec test pulses (see the arrow heads in Control traces). The associated current-voltage relations depict the peak (= T-type + L-type) current (B), L-type current (C) and T-type current (D), measured in control solution (open circles), in the presence of 500 nM TRH (solid circles), and following recovery at 5 min (open triangles). Current traces have been corrected for linear leak and capacitance current in this and succeeding figures. In figures 1 and 3, GTP$\gamma$S was added to the patch pipet solution at a concentration of 20 $\mu$M, although we could not know the actual concentrations of GTP$\gamma$S within cells. Throughout this experiment, $GH_3$ cells were bathed in $Na^+$-free, $Ca^{2+}$-TEA solution to isolate $Ca^{2+}$ currents, and TTX was added to suppress current through $Na^+$ channels.

increases in the L currents were observed (arrow), indicating that the cytosolic GDP$\beta$S markedly blocked the effect of TRH (500 nM) to produce both an increase of T current and a decrease of L current as seen in Fig. 1. This corresponds to the control experiment of Fig. 1.

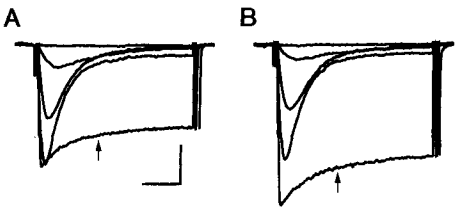


Fig. 2. Cytosolic GDP$\beta$S, an inactivator of G protein, blocked the effect of TRH as seen in Fig. 1. GDP$\beta$S (100 $\mu$M) was rendered within the cell via a patch pipet in (A) and (B). Superimposed $Ca^{2+}$ current traces in (A) and (B) were evoked with test pulses (160 msec duration) spaced by 10 mV to between -50 and -20 mV, and +10 mV (arrows) from a holding potential of -80 mV. Note that in the presence of the cytosolic GDP$\beta$S, T currents in control traces (A) remained largely unchanged by the addition of 500 nM TRH (B), although some increase in the L-current was observed (B, arrow). Vertical and horizontal calibration bars represent 50 pA and 40 msec, respectively.

**Effect of a lowered concentration of TRH (5 nM) on T currents.**

In Fig. 3, control current traces marked with open triangles, are shown superimposed on current traces taken 30 sec after the application of 5 nM TRH (arrows), measured from the same cell. Control current traces show pure T currents evoked by test pulse in the range of -50 to -30 mV and L currents together with T currents by test pulses at -20 and 10 mV, from a holding potential of -80 mV. A reduced concentration of TRH (5 nM) produced significant increases in the amplitude of T current, similar to those induced by 500 nM TRH (Fig. 1). The increase of T current was by 35 ± 6.0% (mean ± S.E.M.; N=6) at the test potential of -30 mV. The appreciable increase of T current by TRH (5 nM) occurred at test potentials in the range of -40 and -10 mV, with no change in the threshold potential of about -60 mV and

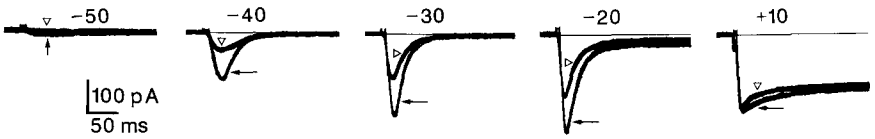


Fig. 3. A lowered concentration of TRH (5 nM) markedly increased the amplitude of the T current, whereas no appreciable change of the L current was seen. Control current traces marked with open triangles are shown superimposed on current traces taken 30 sec after the application of TRH (arrows), in the same cell. From a holding potential of -80 mV, test pulses to between -50 and -30 mV as indicated above each trace induced T-type current, and test pulses at -20 and 10 mV produced both L-type current and T-type current. Six of 7 cells examined showed significant increases of the T currents by 35 ± 6.0% (mean ± S.E.M.; N=6) at the test potential of -30 mV in response to TRH application.

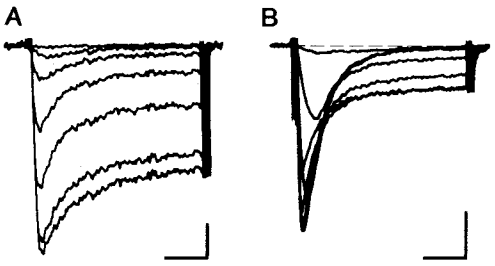


Fig. 4. Intracellularly applied GTP$\gamma$S with a patch pipet containing 50 $\mu$M GTP$\gamma$S induced both an increase of T current and a decrease of L current in (B), through the activation of a G protein, and hence mimicked the effects of extracellularly applied TRH (500 nM) shown in Fig. 1. Families of superimposed $Ca^{2+}$ current traces were obtained from a cell without GTP$\gamma$S (A) and a cell with GTP$\gamma$S (B). No TRH was present extracellularly. Currents were evoked with test pulses (160 msec duration) spaced by 10 mV to between -50 and +10 mV for (A) and (B) from a holding potential of -80 mV. Note that the $Ca^{2+}$ channel current was largely inactivating T current in (B). (A) and (B) show data from different cells. The mean ratio of the amplitudes of T current measured at a test potential of -20 mV relative to the amplitudes of L current at 10 mV was 2.4 (N=3) in a cell with 50 $\mu$M GTP$\gamma$S, in contrast to the reduced mean ratio of 1.0 in a cell without GTP$\gamma$S (N=4, data not shown). Vertical and horizontal calibration bars represent 50 pA and 40 msec, respectively, for (A) and (B). A broken line in (B) indicates 0 current level.

the peak position of about -20 mV (data not shown). However, this lowered concentration of TRH (5 nM) failed to induce a change in the amplitude of L current, as seen at a test potential of 10 mV.

When hydrolyzable GTP (20 $\mu$M) was rendered within cells, instead of non-hydrolyzable GTP$\gamma$S, TRH (5 nM) appeared to give similar effect on the T currents within 5 min after the rapture of the membrane seal. This increase of T current was by 27 $\pm$ 2.0% (N=3) at the test potential of -30 mV.

**GTP$\gamma$S (50 $\mu$M) mimicked the effects of TRH on T- and L-currents.**

Figures 4A and 4B show representative of superimposed $Ca^{2+}$ current traces obtained from a cell without GTP$\gamma$S and a cell with GTP$\gamma$S (50 $\mu$M), respectively. The current traces were elicited by test potentials in the range of -50 to +10 mV in 10 mV increments (160 msec duration) from a holding potential of -80 mV. Despite the absence of extracellular TRH, 50 $\mu$M GTP$\gamma$S produced both an increase of T current and a decrease of L current (Fig. 4B), with no significant change in the threshold potentials of both currents (N=3, data not shown). Hence, the elevated concentration of GTP$\gamma$S (50 $\mu$M) applied internally with a patch pipet was found to mimic the effects on the T- and L-type $Ca^{2+}$ currents induced by externally applied TRH (500 nM). These suggest that the effect of TRH (500 nM) is mediated by a G protein.

## DISCUSSION

The present experiments revealed for the first time that TRH (500 nM) significantly augments T-current amplitude, through the activation of a G pro-

tein, in $GH_3$ cells under whole-cell voltage-clamp conditions. This fact is especially significant for a secreting cell such as the $GH_3$ cell, in which enhancement of the frequency of $Ca^{2+}$ action potentials is a prerequisite for increasing and sustaining the level of $[Ca^{2+}]_i$, which results in tonic hormone secretion (for a review see 15). The increase in the amplitude of T current that we observed in this study may result in the enhancement of $Ca^{2+}$ action potential frequencies in $GH_3$ cells, possibly concomitant with the depression of $K^+$ currents by TRH (4,5). This effect of TRH on T current was mimicked by intracellularly applied GTP$\gamma$S and was blocked by intracellularly applied GDP$\beta$S, suggesting that a G protein is involved in the signal transduction mechanism that links TRH receptor activation to the modulation of T-type $Ca^{2+}$ channels.

The increase in T-current amplitude was found to be accompanied by a reversible decrease in L-type $Ca^{2+}$ current amplitude in response to TRH (500 nM). This reduction in L-current amplitude in contrast to the increase in T-current amplitude, makes it likely that the $Ca^{2+}$ channel current during the prolonged enhanced $Ca^{2+}$ action potential firing induced by TRH is largely T current. Hence it is possible that the net amount of calcium influx is reduced because of the inhibition of L-type current, but that the accumulated intracellular $Ca^{2+}$ level is increased because of the increased frequency of action potentials as suggested by (16). Furthermore, an accurate regulation of $[Ca^{2+}]_i$ could be possible by changing frequencies of $Ca^{2+}$ entry largely through T channels, whose amount may be relatively uniform and insensitive to alteration in action potential waveform (9).

In accordance with the present findings using whole-cell recorded L currents, Levitan and Kramer (8) have recently shown that single L-type $Ca^{2+}$ channel activity from $GH_3$ cells is dramatically inhibited by TRH (100 nM), using a new patch clamp configuration named the "perforated vesicle method", in which the vesicle retains cytoplasm and contains ion channels in an outside out orientation, indicating that TRH reversibly decreased the probability of a L channel being open without affecting single L-channel conductance; the single channel conductance is 32 pS in 110 mM barium. In conventional whole-cell recordings or out-side out patches in which crucial cytoplasmic components have apparently been lost, TRH has no effect on L type $Ca^{2+}$ channels (4,20). The present result that L currents recorded in conventional whole-cell configuration may be successfully inhibited by TRH (500 nM), was obtained using patch pipet solution supplemented with GTP$\gamma$S (20 $\mu$M).

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