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# Effects of hypothalamic peptides on electrical activity and membrane currents of 'patch perforated' clamped $GH_3$ anterior pituitary cells

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'Perforated-patch' recordings of rat anterior pituitary $GH_3$ cells allow long and stable monitoring of electrical activity and membrane currents. Under current clamp conditions, the biphasic effect of thyrotropin releasing hormone (TRH) consisting of a transient hyperpolarization followed by a longer phase of increased action potential frequency is fully preserved. Somatostatin suppresses action potential activity and antagonizes the second phase of enhanced spiking caused by TRH. Voltage clamp records of isolated currents indicate that TRH affects calcium-dependent potassium currents, but does not alter either voltage-dependent potassium or calcium currents at times and concentrations at which the electrical activity is increased.

Perforated-patch recording; Thyrotropin releasing hormone; Somatostatin; Anterior pituitary

## 1. INTRODUCTION

Electrophysiological recordings from $GH_3$ cells using conventional high resistance micro-electrodes have revealed that somatostatin (SS) depresses [1] and TRH enhances cell excitability. The effect of TRH is quite complex, consisting of an initial phase of transient hyperpolarization [2–5] followed by a second phase in which membrane conductance is decreased while both the rate of production and the length of APs are increased [2,3,5–7]. This second phase causes a plateau of elevated intracellular $Ca^{2+}$ due to influx of extracellular $Ca^{2+}$ ([8–10] for review see [11]) and coincides with a sustained increase in secretion [9,12–16]. The importance of $Ca^{2+}$ in this sustained secretion is controversial [9,11,12], and it has been suggested that this cation is only necessary at low TRH concentrations when the increases in diacylglycerol are submaximal [16]. The reasons for the increased production of APs and what concrete conductances are implicated in this effect are not well known. Voltage clamp recordings using the whole-cell configuration of the patch-clamp technique have revealed an attenuation of voltage-dependent $K^+$ currents by TRH [17]. However, it is improbable that a reduction of voltage-dependent $K^+$ conductance could bring about all the changes in membrane parameters reported in $GH_3$ cells [18]. On the other hand, we and others [4,19] failed to consistently detect the delayed increase in AP frequency under whole-cell current clamp. This result as well as the prominent rundown of membrane currents and cell excitability caused by the internal dialysis [4] indicate that some important constituents of the cellular response are lost during whole-cell recordings. In fact, addition of a cytosolic extract to the pipette solution prevented the rapid rundown of the initial response to TRH, but the second, maintained phase of activity still could not be consistently obtained [4]. In this report, the 'perforated-patch' configuration of the patch-clamp method [20] has been used to preserve the response of $GH_3$ cells to TRH and SS under current clamp. The combination of this technique with pharmacological methods previously employed to isolate voltage- and $Ca^{2+}$-dependent $K^+$ currents [18,21–23] and voltage-dependent $Ca^{2+}$ currents [21,24,25] in these cells has also been used to study the participation of these individual current types in the effects of TRH.

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*Abbreviations:* TRH, thyrotropin releasing hormone; SS, somatostatin; $V_p$, pipette potential; TEA, tetraethylammonium; 4-AP, 4-aminopyridine; $I_{K_v}$, voltage-dependent $K^+$ current; AP, action potential

## 2. MATERIALS AND METHODS

Nystatin, TRH, SS and TEA were purchased from Sigman (St. Louis, MO). 4-AP was from Aldrich (Germany). $GH_3$ cells (ATCC CCL 82.1) were a gift of Dr G.J. Kaczorowski (Merck, Sharp & Dohme Res. Lab., Rahway, NJ, USA). Cells were plated in 35 mm tissue culture plastic dishes containing Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham supplemented with 15% horse serum, 2.5% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin (Sigma). The cells were grown at 37°C in a humidified atmosphere of 95% air and 5% $CO_2$ for 5–10 days before they were used for experiments. Recordings were made at room temperature (20–25°C) in a recording chamber of 0.2–0.3 ml. The chamber was continuously perifused at 1 ml/min using an extracellular-like solu-

tion containing (in mM): 140 NaCl, 1 KCl, 1 $MgCl_2$, 10 glucose, 10 $CaCl_2$ and 10 Hepes titrated to pH 7.3 with NaOH. Perforated-patch recordings [20] were performed using a List EPC-7 (List Electronics, Darmstadt, Germany) patch-clamp amplifier. 1-3 MΩ electrodes were fabricated from Boralex disposable micropipettes (Rochester Scientific, Rochester, NY, USA). The tip of the pipette was initially filled with nystatin-free high $K^+$ solution containing (in mM): 65 KCl, 30 $K_2SO_4$, 10 NaCl, 1 $MgCl_2$, 50 sucrose, 0.5 EGTA and 20 Hepes titrated to pH 7.3 with KOH. The remainder of the pipette was then backfilled with the same solution also containing 0.25 mg/ml nystatin added from a stock of 50 mg/ml nystatin dissolved in dimethylsulphoxide. In experiments designed to study $Ca^{2+}$ currents, $Cs^+$ replaced $K^+$ in the pipettes and sometimes 1 μM tetrodotoxin was also included in the extracellular solution. The course of perforation was followed monitoring the progress of capacitive transients and evoked membrane currents after occasional switching to voltage-clamp mode from current-clamp mode. 10-30 min were usually enough to yield acceptable levels of membrane permeabilization. Current-clamp records of $V_m$ stored on video tapes and voltage-clamp records of membrane currents were analyzed using an Atari computer and commercial software (Instrutech Corp., Elmont, NY, USA). APs' frequency was determined in periods of at least 80 s either before or 2-5 min after addition of TRH. The characteristics of the spikes were quantified randomly selecting ten APs and averaging the value of the selected parameter. Current records were digitally filtered at 500 Hz and plotted using a Hewlett-Packard 7475A plotter. Leak and capacitive currents were subtracted on-line using a P/4 procedure. Solution junction potentials were compensated for in each experiment. Data are expressed as mean ± SE with the number of cells in parentheses.

## 3. RESULTS AND DISCUSSION

Electrical activity was recorded in 48 patch-perforated current-clamped $GH_3$ cells showing fully developed action potentials. In some additional cells, the activity only consisted of small, subthreshold potential oscillations of several mV in amplitude which do not originate any full spike. These cells and those in which no activity could be evoked by injection of considerable amounts of depolarizing current were discarded. Given the variability of time and extension of perforation of the patches, no detailed statistics on percentage of active vs inactive cells were obtained. The mean resting potential averaged 36 ± 0.8 mV ($n=48$). The all-or-none APs displayed positive overshoots (mean value 5.5 ± 1.0 mV ($n=48$)) and clear after-potentials but varied in duration (at half-amplitude) from cell to cell. In most of the cells the duration of the spikes was short, averaging 29 ± 3.8 ms ($n=35$; a typical time course of short duration spikes is shown in the lower part of Fig. 1). 27% of the cells showed APs longer than 100 ms (mean value 182 ± 22 ms ($n=13$)). The cause of this variability is not known, but very probably reflects differences in properties of the cell membrane in different individual cells since: (i) the characteristics of the APs were minimally altered during recordings lasting for tens of minutes when no changes of experimental conditions were made; and (ii) variations in the amount of current injected to evoke the electrical activity produced considerable changes in the firing rate, but minimally modified the shape of the spikes in a given cell. On the other hand, we did not find significant differences in response to hormones (see below) in cells showing different durations of the action potentials.

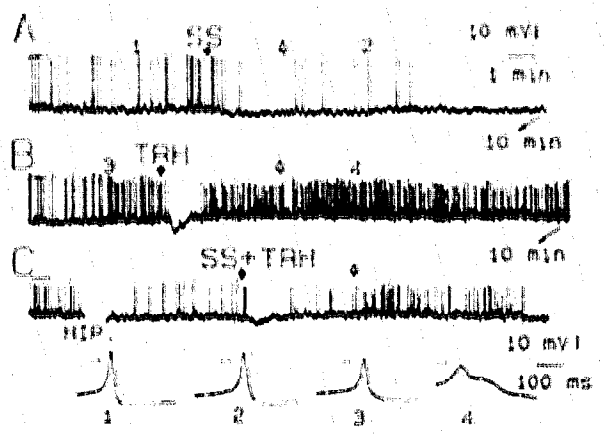


Fig. 1. Effect of SS and TRH on electrical activity in patch-perforated current clamped $GH_3$ cells. Closed arrows indicate the beginning of perifusion with 1 μM hormones. Open arrows indicate the beginning of washout. Traces have been interrupted for 10 min as indicated. 'HIP.' indicates application of a small hyperpolarizing current. Sampling rates of 10 ms/point were used. Four APs recorded at 0.1 ms/point are shown in an expanded time scale at the bottom. 0 mV is represented by horizontal lines.

Fig. 1 shows that addition of SS to patch-perforated current-clamped cells caused a drastic reduction of electrical activity. The effect lasted for several minutes after switching the perifusion to a hormone-free medium, but it was completely reversible upon exhaustive washout. Similar results were obtained in three additional cells. Fig. 1 also shows that the response to TRH in the same cell was biphasic, with a transient hyperpolarization after a short delay of several seconds, followed by a longer period in which AP frequency was augmented. These two phases were observed in 54% of the 35 cells treated with TRH. 15% and 18% of the cells responded with only the first or the second phase, respectively. Finally, we dit not detect any response to the hormone in 13% of the cells. These results resemble those obtained with intracellular microelectrodes ([1-7], see also [10]). Previous analysis of the electrical response to TRH by combination of voltage and current clamp has been constrained by the inability to evoke the second phase of enhanced AP frequency using conventional whole-cell recording ([4,19], and data not shown). Thus, we focused on this second phase using the patch-perforated current clamped cells. The increase in frequency of firing was accompanied by a decrease in mean resting potential of 2.7 ± 0.5 mV (in 35 cells treated with different concentrations of TRH) and a clear modification of the shape of the spikes: The overshoot was decreased by 2.1 ± 0.8 mV ($n=24$) and both the raising and the repolarization phases were slowed (see inset at the bottom of Fig. 1) yielding APs with durations 1.45 ± 0.12-fold ($n=24$)

longer than those recorded before addition of TRH. The enhancement in frequency was partially reversed after 10 additional minutes of perifusion without hormone. Fig. 1C shows that SS not only suppresses AP activity, but also antagonizes the second phase of increased frequency in response to TRH. This result is not due to inability of TRH to evoke its effects when added twice to the same cell because: (i) although reduced in size, the transient hyperpolarization was still detected upon challenge of the cell with the hormones; (ii) removal of the hormones produced a faster recuperation of the activity as compared with the long delay necessary to restore activity upon SS washout; and (iii) addition of TRH alone at later times was able to induce the second phase of increased spiking (not shown).

Table I shows that the increase in frequency elicited by TRH is dependent on the dose of hormone. The response starts at concentrations around 1 nM and shows a maximum at ca 10 nM, a value close to the $K_d$ of TRH for its receptor and to the $EC_{50}$ for stimulation of secretory responses in $GH_3$ cells [13]. Addition of 10 $\mu$M TRH (a very high oversaturating concentration of the hormone) increased frequency only in two of the five cells tested. In two additional cells, the activity decreased by 20% and in one cell APs were abolished after the transient hyperpolarization. It is interesting to note that the initial hyperpolarization is small (if any) at concentrations of TRH between 1 and 10 nM and that its size is increased when the amount of TRH is raised. On the other hand, there is no decrease in the magnitude of this response up to concentrations of 10 $\mu$M (not shown). However, 50 ± 4 s ($n=6$) are necessary to recover the cells from the hyperpolarization at 10 nM TRH, but only 25 ± 4 s ($n=4$) at 10 $\mu$M TRH. The fact that the second phase of enhanced AP frequency can still be evoked in perforated-patch clamped cells is especially relevant since the enhanced production of APs should be a crucial factor for the sustained elevation of intracellular $Ca^{2+}$ and for the second phase of sustained secretion. However, the role of $Ca^{2+}$ influx for sustained secretion is controversial [9,11,12]. Differences in concentration of the hormone can account, at least in part, for these discrepancies (cf. 100 nM in [9], and 1 $\mu$M in [12,16]; see also [11]).

The concrete ionic currents implicated in the second phase of TRH effects are not well known. Addition of mM amounts of TEA to the extracellular medium blocks most of the $Ca^{2+}$-dependent component of the outward $K^+$ currents [18,21–23] and unmasks a voltage-dependent inactivating current ($I_{K_v}$) (Fig. 2A). This current corresponds to that previously identified as $I_{K_v}$ under whole-cell voltage clamp [18,21–23] since: (i) it is not sensitive to extracellular TEA up to 30 mM; (ii) it activates at voltages above -40 mV and shows activation and inactivation kinetics which coincide with those of $I_{K_v}$ (Fig. 2Ab,c; see also [18,21-23]); (iii) the steady state voltage dependence of inactivation indicates that the current half-inactivates at approximately -30 mV; (iv) recovery from inactivation at -70 mV is a slow process which takes 3–6 s for 50% and around 15 s for essentially complete recuperation of the current; and (v) it is nearly abolished by addition of 5 mM 4-AP to the bath (cf. see Fig. 2B). Application of 100 nM TRH causes no changes in this current measured at various membrane potentials (Fig. 2A). Identical result was obtained in 10 additional cells at 100 nM and in 3 cells at 1 $\mu$M TRH (not shown). The lack of effect on $I_{K_v}$ contrasts with the clear variations detected on $Ca^{2+}$-dependent $K^+$ currents. The inactivation of $I_{K_v}$ and the presence of 5 mM 4-AP in the medium produce outward currents which almost exclusively consist of slowly rising $Ca^{2+}$-dependent $K^+$ currents at the end of a 200–400 ms depolarizing voltage step (Fig. 2Bb; see also [18,21–23]). This fact is further supported by the N-shape of the $I$ vs $V$ plot of the current (Fig. 2Bc). Furthermore, the current is 80–95% reduced at all voltages in the range from -20 to +80 mV by addition of 5 mM $Co^{2+}$ to the bath or by substitution of the medium with one from which $CaCl_2$ has been omitted. These treatments also eliminate the N-shape of the $I$ vs $V$ plot. As shown in Fig. 2B, addition of TRH caused a small increase in this current during the first 30 s following the application of the hormone (mean increase 9.3% ± 1.1; $n=9$). This increase was detectable in 9 of the 12 cells treated with TRH and correlated in time with the transient hyperpolarization (see above). The increase was followed by a period of several minutes in which the current was decreased (mean decrease 17.5% ± 2.8; $n=11$). The reduction and the subsequent recuperation of the current was usually accompanied by a change in the shape of the $I$ vs $V$ plot even at times at which recuperation of the current is almost complete. The more consistent change was a 10–20 mV shift in the minimum of the curve towards the left (six cells). The direction of the shift and the fact that capacitive transients remained unaltered (not shown) indicate that series resistance variations are not implicated in these effects. In fact, sometimes the minimum of the curve is raised (two cells), see Fig. 2Bc)

Table I

TRH concentration dependence for modification of AP frequency

| [TRH] (M) | AP frequency (%)* |
|---|---|
| $10^{-9}$ | 110 ± 7 ($n=2$) |
| $10^{-8}$ | 220 ± 32 ($n=7$) |
| $5 \times 10^{-8}$ | 200 ± 10 ($n=2$) |
| $10^{-7}$ | 196 ± 30 ($n=6$) |
| $10^{-6}$ | 135 ± 7 ($n=4$) |
| $10^{-5}$ | 138 ± 55 ($n=5$) |

*Percentages refer to the same cells before treatment with TRH. Values of AP frequency in untreated cells were 0.19 ± 0.02 Hz for $10^{-9}$, $10^{-8}$ and $10^{-7}$ M, 0.22 ± 0.05 Hz for $5 \times 10^{-8}$ and $10^{-6}$ M and 0.34 ± 0.05 Hz for $10^{-5}$ M

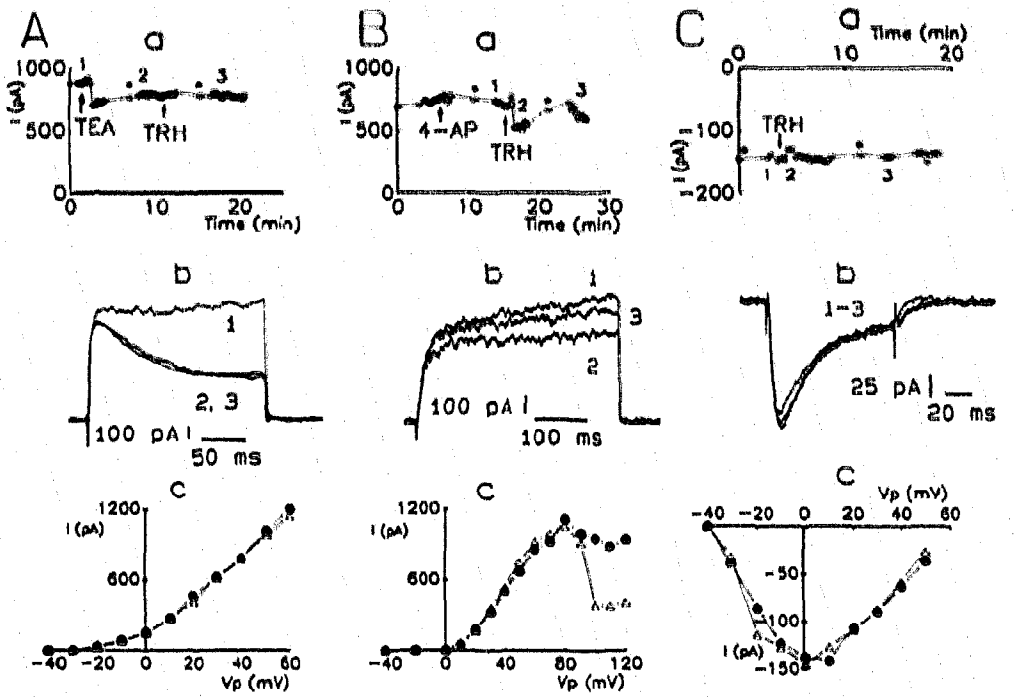


Fig. 2. Effect of TRH on $I_{K_v}$ (A), $Ca^{2+}$-dependent $K^+$ currents (B) and $Ca^{2+}$ currents (C). (a) Time courses of maximum outward and inward currents evoked by depolarization steps to +40 (A), +50 (B) and 0 (C) mV from a holdig potential of -70 mV. (b) Representative current traces obtained at the times indicated by numbers in panels (a). Note differences in duration of the depolarization steps. (c) *I* vs *V* plots recorded at the times indicated by asterisks in (a), before (triangles) and after (circles) addition of TRH. TEA and 4-AP were used at 5 mM. TRH was perifused for a period of 2 min starting at the arrow at concentrations of 50 nM in (C) and 100 nM in (A) and (B). Pipettes did not contain EGTA in (B). Note that generation of the *I* vs *V* plot overlaps with the period of recuperation of the current in (B).

which further suggests that background $Ca^{2+}$ remains elevated in the cell. These variations most likely represent true effects on $Ca^{2+}$-dependent $K^+$ currents since not only $I_{K_v}$ but also voltage-dependent $Ca^{2+}$ currents remain unaffected after addition of TRH. Substitution of $K^+$ by $Cs^+$ into the pipette minimizes the contribution of $K^+$ currents to total cellular current. Given the rapid inactivation of the $Na^+$ currents [24,25], the resultant inward currents must consist exclusively of $Ca^{2+}$ currents after a few ms of depolarization. In any event, inward currents showed minimal washout up to 60 min of recording and were not significantly affected by TRH (8 cells, see Fig. 2C). The only detectable effect in this case was a slight and sporadic reduction of the current during the first minute of treatment with the hormone. This reduction never exceeded 10% of the total current and most likely is not a true reduction in inward current, but rather a decrease due to the transient increase in outward current carried either by $Cs^+$ or by residual intracellular $K^+$ through $Ca^{2+}$-dependent $K^+$ channels (see above).

Our results demonstrate that hormonal responses and membrane currents which are rapidly diminished using the whole-cell configuration of the patch-clamp technique, are fully preserved when perforated patches are used. The possibility of combined voltage- and current-clamp analysis in the same cells is another remarkable quality of the perforated-patch technique. TRH induced reductions on $I_{K_v}$ have been previously reported in whole-cell mode [17]. However, we could not detect any effect of the hormone on this current. The reason for this discrepancy is not known. Reductions in $Ca^{2+}$-dependent currents shorter than those reported her have also been previously shown [17], but they have been interpreted as due to a decrease in total outward current secondary to reductions in $I_{K_v}$. The inhibitions shown in this report cannot be attributed to this fact since: (i) no changes in $I_{K_v}$ were detected under our experimental conditions; and (ii) $Ca^{2+}$-dependent $K^+$ currents were recorded in the presence of concentrations of 4-AP that largely abolish $I_{K_v}$. It is important to emphasize that the reductions on $Ca^{2+}$-dependent $K^+$ currents extend for a period of several minutes well into the time required to enhance the frequency of spikes. This suggests that changes in these currents can contribute to the sustained effects of TRH. However, their relevance for sustained elevations of $Ca^{2+}$ and secretion is difficult to evaluate. In fact, no spikes can be produced under the voltage-clamp conditions used here, but sometimes the N-shape of the *I* vs *V* plot is modified after TRH (see above), suggesting that background $Ca^{2+}$ has been elevated in the cell. Since more than one current component contributes to the total $Ca^{2+}$-dependent $K^+$ currents [22,26], an exact

knowledge of the concrete conductance(s) modified by TRH awaits further investigation. On the other hand, further experiments are also necessary to know whether voltage-independent influx of $Ca^{2+}$, $Ca^{2+}$ liberation from cellular stores, failure to pump $Ca^{2+}$ outside the cytoplasm or a combination of several factors contribute to the effects observed in response to TRH.

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