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Rundown of GH3 Cell K^+ Conductance Response to TRH Following Patch Recording Can be Obviated with GH3 Cell Extract

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SUMMARY: GH3/B6 pituitary cells release prolactin (PRL) in response to thyrotropin releasing hormone (TRH). Electrophysiological assays of individual GH3 cells with sharp high-resistance microelectrodes have revealed complex effects of TRH on membrane excitability consisting of a transient hyperpolarisation (1), which is thought to result from activation of Ca-dependent K^+ conductance (2), followed by a prolonged phase of spontaneous, Ca-dependent action potential activity (3). Using the whole-cell patch recording (WCR) technique (4), we have found that these TRH actions on GH3 excitability rapidly rundown following patch recording. When the supernatant from osmotically lysed GH3 cells was added to the WCR patch pipette, the K^+ conductance response was not only promoted but well-maintained. The results indicate that diffusible factors mediate these TRH actions and further, that the WCR technique should be useful in identifying different second messengers and elucidating their roles in membrane excitability and PRL secretion.

TRH has well-established effects on cytoplasmic and membrane activities of GH3 cells. Spectrofluorometric assays using Ca indicators have shown that TRH increases intracellular Ca^{2+} levels $[Ca^{2+}]_i$ in two phases: a transient 10-20 fold increase in $[Ca^{2+}]_i$ that is thought to involve mobilization of Ca^{2+} from an internal pool (5) followed by a sustained increase of lower amplitude and longer duration, which is considered to reflect Ca^{2+} entry through voltage-activated membrane channels (6,7). Rapid alterations in inositol lipid metabolism leading to the production of inositol triphosphate (IP3) and diacylglycerol have also been reported following TRH stimulation (8-11). Since IP3 mobilizes Ca^{2+} from organelle stores in a variety of cell phenotypes, it has been hypothesized that IP3 mediates the observed increase in $[Ca^{2+}]_i$ (10-12). Electrophysiological recordings from individual GH3 cells using conventional, high resistance (80-100 meghoms)

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micro-electrodes have consistently revealed complex actions of TRH on cell excitability in the majority of cells recorded. The most common actions include a transient hyperpolarization of the cell typically lasting 10-30 seconds, which is associated with an increase in membrane conductance. This phase of membrane activity is thought to involve an increase in K^+ conductance that is mediated in some way by Ca^{2+} ions (1,2,13,14,15), since it can be mimicked by extra-cellular application of the Ca ionophore A23187 and eliminated by including calcium chelators in the microelectrode recording solution. The Ca-activated K^+ conductance response is often followed, but not always, by the appearance of spontaneous Ca-dependent action potentials at the level of the resting membrane potential (1,3,13,14,15). This biphasic response to TRH can be evoked repeatedly at a low frequency of stimulation, but desensitizes if the applications are too frequent.

The recently developed whole-cell recording technique with patch electrodes offers a number of advantages over the conventional intracellular recording method; however, the rundown of excitability consistently reported in these (14,16) and many other cells, represents serious limitation of the assay. In this report, we show that the well-characterized electrophysiological response of GH3 cells to TRH also rundowns within several minutes after rupture of the membrane for whole-cell recording and then demonstrate that the response to TRH can be maintained by using an aqueous extract of osmotically lysed GH3 cells to formulate the recording solution in the patch micropipette internally dialyzing the cell. This strategy may help to elucidate the second messenger mechanisms involved in peptide-evoked electrical signals at the single-cell level of sensitivity.

Materials and Methods

GH3/B6, a sub-clone of the GH3 cell line, was originally obtained from Dr. A. Tixier-Vidal (College de France, Paris). Cells were cultured in Ham's F10 nutrient medium supplemented with 15 percent horse serum and 2 percent fetal calf serum and maintained at 37°C in a humidified

atmosphere with 5 percent CO_2. Electrophysiological recordings were conducted on low-passage (10-20) cells 5-10 days after replating. The extracellular salt solution consisted of (in mM): 150 NaCl, 5 KCl, 10 $CaCl_2$, $2MgCl_2$, 5 glucose and 5 HEPES. The pH was adjusted to 7.2-7.3 and osmolarity to 300-310 mOsm.

Electrophysiological studies of GH3 cells were performed using the whole-cell recording (WCR) configuration of the patch-clamp technique (6) with blunt "patch" electrodes. Patch pipettes had resistances of 3-5 meghoms when filled with the following solution (in mM): 140 K-gluconate, 2 $MgCl_2$, 1 EGTA, 5 HEPES dissolved in deionized water (pH 7.3). Additional reagents, as indicated below, were added to modify this recording solution. The experiments were carried in current- and voltage-clamp configuration, using a DAGAN 8900 amplifying system. Experiments were performed at room temperature (22-25°C). We attempted to maintain the electrical response to TRH by making an aqueous extract of the GH3/B6 clone. This was done by osmotically lysing the clone in distilled water, then using the supernatant to formulate the K^+-gluconate recording solution in the patch electrode. The aqueous extract of GH3/B6 cells was made as follows: 1.5×10^6 cells were allowed to lyse for 15 min. in the presence of 1 ml distilled water. The protein constant of the cellular extract as evaluated by the method of Lowry was 0.4 mg protein/ml. The supernatant was carefully collected, filtered through a 0.45 micron millipore filter and then used to prepare the K^+-glu recording solution.

Results and discussion

Electrical responses to TRH using a conventional intracellular recording method with a sharp-point high-resistance (80-100 meghoms) microelectrode (Fig. 1A1) are qualitatively different from those obtained using whole-cell patch clamp recording with blunt pipettes (3-5 megohms) (Fig. 1A2). We have found that using patch pipettes the biphasic electrical response to TRH disappears within minutes of initiating the WCR (Fig. 1B) or completely fails to appear in one-third of the cells recorded (14/40). These latter results have not been included in the statistics illustrated in Fig. 2B since we have no evidence that these cells actually express functional receptors for TRH. The fading is not due to desensitization since restoration has never been observed during recordings lasting up to 1 hr, even when TRH was applied at infrequent intervals. Rundown of TRH-induced changes in excitability was observed whether the patch recording solution contained K^+ gluconate or KCl. Similar rapid loss of an outward, presumably K^+ current response and associated increase in conductance to TRH was recorded under voltage clamp in WCR utilizing the standard K^+ gluconate recording solution (Fig. 1B1).

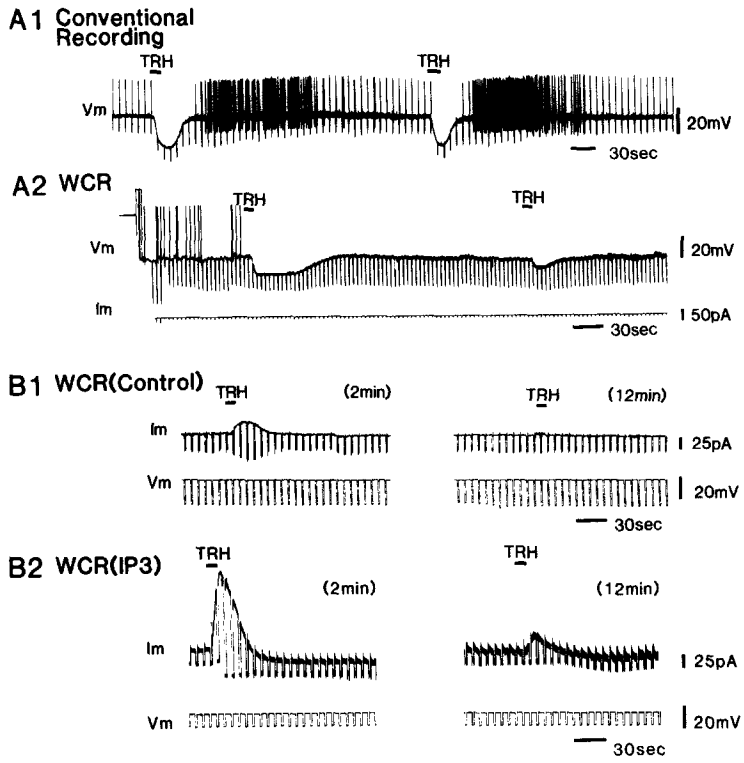

Fig. 1: The effects of TRH on the excitable membrane properties of GH3 cells rapidly disappear using patch clamp recording techniques.
A1: The effects of TRH on GH3 cell membrane excitability have been studied using intracellular recording techniques with sharp-point, high-resistance microelectrodes (80-100 megohms) filled with 3M KCl. With this technique 50nM TRH induces reproducible effects consisting of a transient hyperpolarization followed (in this but not all cells) by generation of spontaneous action potential activity.
A2: Effect of TRH on the membrane potential of a GH3 cell using the whole-cell recording configuration of the patch-clamp technique (4) with blunt, patch electrodes. Under these conditions, 50nM TRH induces significant hyperpolarization of the cell shortly after impalement but this response fades rapidly with time.
B1: Under voltage clamp (V_h =-40 mV) 50nM TRH induces an outward current response associated with an increase in membrane conductance shortly after the intracellular recording is initiated (0-2 min). The amplitude of the TRH-induced current response and associated conductance is markedly attenuated after 12 minutes recording.
B2: The cell was recorded using K^+-gluconate solution containing 10 micromolar IP3. The TRH-induced outward current response and associated increase in conductance at -40 mV are noticeably greater during the first two minutes of recording (compare with B1) but rundown quickly.

The rapid rundown of the biphasic response to TRH under these recording conditions suggests that the changes in membrane excitability may be critically dependent on soluble substances that quickly diffuse into the patch pipette after the WCR has been initiated. Since Ca^{2+} ions are thought to mediate TRH-induced activation of K^+ conductance, we buffered $[Ca^{2+}]_i$ over the range 10^{-8} to 10^{-6}M by including

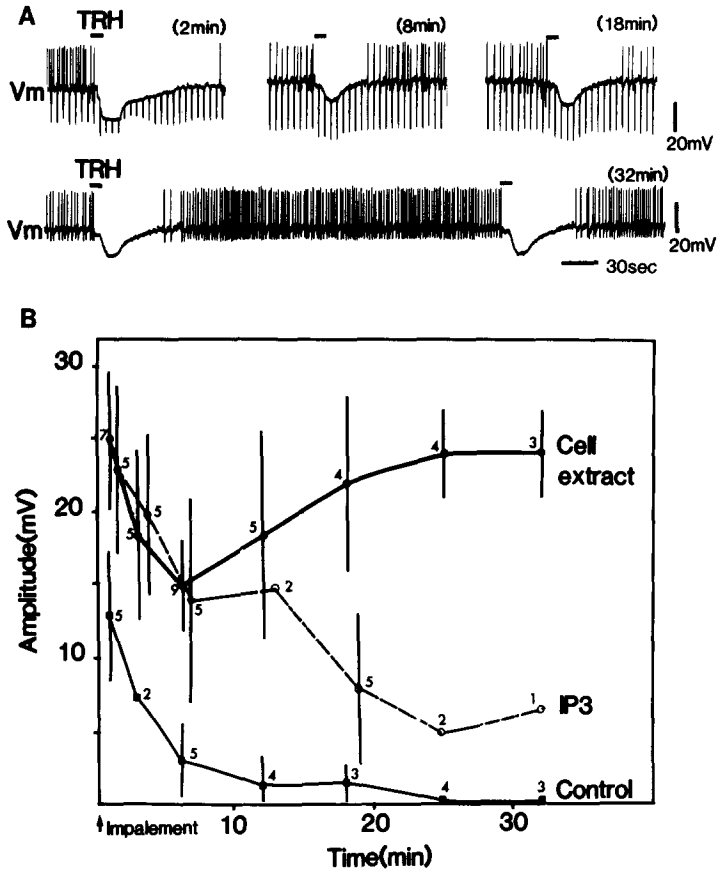

Fig. 2: Hyperpolarizing responses to TRH that rundown in patch clamp recording can be altered by IP3 or aqueous GH3 cell extract.
A. Current-clamp configuration of a whole-cell patch clamp recording from a GH3/6 cell using a pipette filled with an aqueous extract of GH3 cells (0.4 mg protein/ml). Constant-current hyperpolarizing pulses (0.5 nA, 100 msec at 1Hz) injected in the top set of traces illustrate that the hyperpolarizing responses to repeated applications of 50nM TRH are accompanied by an increase in conductance. At 32 minutes recording time the hyperpolarizing response induced by TRH is 18 mV, nearly identical to that recorded initially (19 mV).
B. Time-courses of the amplitudes of hyperpolarizing responses to TRH under different recording conditions using patch pipettes filled with either K^+-gluconate, k^+-gluconate + 10 micromolar IP3 or K^+-gluconate prepared with aqueous extract of GH3/B6 cells. The numbers in parenthesis are the number of cells tested at particular times in the different recording conditions. The vertical bars are standard deviations for data points with a large enough sample.

suitable concentrations of Ca^{2+} ions and Ca^{2+} chelator in the patch pipette (17). Buffering $[Ca^{2+}]_i$ in this concentration range did not prevent rundown of either phase (not shown). Inclusion of adenosine triphosphate (ATP) or and ATP-regenerating system (2mM ATP, 5mM creatine phosphate, 5 units/ml. creating phosphokinase, with or without 10 micromolar antimycin A) was likewise ineffective. Since $[IP3]_i$ rises

rapidly following TRH application, we tested the possibility that it might maintain the membrane response. Inclusion of IP3 in the patch pipette recording solution led to a concentration-dependent increase in resting membrane potential from -50.5 ± 5.3mV (mean ± S.D. for 9 cells recorded with K^+-glu alone) to -54.4 ± 5.3 mV (7 cells recorded with K^+-glu plus 5 micromolar IP3) to -63.4 ± 6.1mV (8 cells; K^+-glu plus 10 micromolar IP3). Under voltage clamp the initial outward current response and associated conductance increase to TRH were consistently greater than control, but this response rapidly faded (Fig. 1B2) with a time course similar to that recorded under control conditions (Fig. 2B). These results indicate that although $[IP3]_i$ increases steady-state membrane potential and promotes TRH-induced K^+ conductance, it cannot sustain the peptide-induced response, nor can it restore the phase characterized by spontaneous action potential activity (unpublished observations).

Since WCR likely dilutes soluble substances from the cytoplasm of recorded cells, we osmotically lysed cells to allow diffusion of soluble components into the extracellular medium and then prepared the standard K^+ gluconate recording solution using such a lysate instead of distilled water. Inclusion of aqueous GH3 cell extract preserved the K^+ conductance response induced by TRH, but the phase of spontaneous activity could still not be detected consistently (Fig. 2A). There were no clear differences in the active or passive membrane properties of cells recorded using the lysate relative to those recorded without extract (unpublished observations).

Thus, to compare the efficacy of the lysate in maintaining the K^+ conductance response with that afforded by the standard recording solution we current-clamped cells at -40mV using differently formulated recording solutions. With the standard K^+-gluconate WCR the amplitude of the TRH-induced hyperpolarization was 13 ± 8.3 mV (mean ± S.D., n = 5 cells) at 1 min. after WCR initiation and 0.5 ± mV (n = 4) after 12 min

(Fig. 2B). Little, if any response could be detected at 25 min. despite otherwise well-maintained resting potential and active and passive membrane properties (not shown). Inclusion of 10 micromolar IP3 in the standard recording solution doubled the initial amplitude of the initial hyperpolarizing response (Fig. 2B) despite the presence of a less resistive membrane. Presumably, activation of K^+ conductance by TRH under these recording conditions completely dominates GH3/B6 membrane properties so that the membrane potential is quickly and effectively held near the reversal potential for the K^+ conductance (-60 to -70mV). However, the amplitude of the hyperpolarizing response to TRH decreased with a time course roughly parallel to that observed during K^+-gluconate recordings, although at recording times longer than 15 minutes an attenuated response could still be detected (Fig. 2B). In contrast, inclusion of an aqueous extract of osmotically-lysed GH3/B6 cells not only doubled the amplitude of the initial hyperpolarization to TRH relative to that seen without extract but also preserved the response for the duration of the WCR (Fig. 2A,B). There was a detectable and consistent transient depression in response amplitude over the first 10-15 minutes to a level similar to that recorded for the initial response when recording with K^+-gluconate alone. TRH-induced hyperpolarization at 25 minutes was similar to the initial response. Extract efficacy was lost upon storage overnight at 4^oC.

Different concentrations of protein in the aqueous extract were also studied. Samples containing 0.1 mg/ml led to results that were not different from those obtained with the standard recording solution (Fig.3). 0.4 mg/ml protein was the most effective concentration at maintaining a stable TRH-induced hyperpolarization, 0.2 mg/ml protein being only partially effective (Fig. 3). Inclusion of a similarly prepared aqueous (0.4 mg/ml protein) extract of C6 glioma cells, which are clonal but neither TRH-responsive nor prolactin/growth hormone-secreting, was ineffective (Fig. 3). The results show that

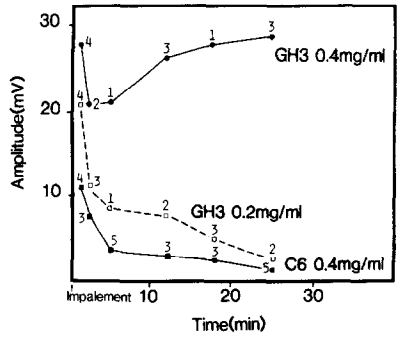

Fig. 3: Dilute aqueous extract of GH3 cells or equi-concentrated extracts of C6 glioma cells do not prevent rundown of the TRH-induced response. Aqueous extract of GH3/6 cells at 0.4 mg/ml was diluted to produce samples having concentrations of 0.1 and 0.2 mg total protein/ml. Samples containing 0.1 mg/ml were not different from those without any extract in terms of their efficacy in supporting TRH-induced hyperpolarization over time. GH3 cells recorded using aqueous C6 cell extract responded to TRH with an initial hyperpolarizing response (11.25 ± 3.2 mV in amplitude; n = 7) similar to that detected without extract but this response faded rapidly with time. More concentrated aqueous extract of C6 cells (0.6 mg protein/ml) was also ineffective.

dilute extracts of GH3/B6 cells and extracts of identical protein concentration from C6 cells could not preserve the peptide-induced response.

In sum, TRH-induced Ca^{2+}-activated K^+ conductance responses, which are attenuated in intensity and rundown in a matter of minutes during patch clamp recordings of GH3/B6 cells, can be intensified but only transiently by including IP3 in the pipette. Aqueous extracts of clonal GH3/B6 but not clonal C6 glioma cells both intensify and maintain the K^+ conductance response. Thus, diffusible factors including IP3 contribute to the peptide-induced K^+ conductance. Patch pipettes afford the opportunity to elucidate the second-messenger mechanisms involved in TRH-evoked changes in excitability in pituitary cells at the single-cell level of sensitivity.

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