Thyrotropin Releasing Hormone Stimulation of Prolactin Release

EVIDENCE FOR A MEMBRANE POTENTIAL-INDEPENDENT, Ca2+-DEPENDENT MECHANISM OF ACTION* 

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Thyrotropin releasing hormone (TRH) and high extracellular K+ induce prolactin release from rat pituitary cells (GH3 cells) in culture. TRH, as well as high K+, increased Ca2+ efflux and stimulated prolactin release simultaneously. High K+, which also increased thyrotropin secretion may involve Ca2+ as a coupl agent in a manner similar to that for release of secretory products from a number of tissues (1, 2). Stimulated release of thyrotropin and prolactin from pituitary cells in vitro has been shown to be dependent upon Ca2+ in the incubation medium (3, 4). Manipulations which increase the concentration of cytosolic Ca2+ such as incubation of pituitary cells with ionophores (5) or high K+ (6, 7), increase hormone release. In electrophysiological studies, Ca2+ action potentials generated by pituitary cells have been shown to be dependent upon Ca2+ in the incubation medium (3, 4). Since the intracellular metabolism of Ca2+ is distributed across the plasma membrane in the absence of any detectable change in the resting membrane potential. Since action potentials generated by these cells were reduced or abolished by a Ca2+ channel blocker and only slightly changed in Na+-free medium, it was concluded that they were generated by a Ca2+ mechanism. These data suggest that after TRH, Ca2+ may be mobilized across the plasma membrane in the absence of any change in membrane potential.

In this report we have measured simultaneously the effect of TRH on the membrane potential and on Ca2+ efflux and prolactin release from rat pituitary cells of the GH3 strain. Membrane potential was measured indirectly by use of the lipid-soluble cation triphenylmethyl phosphonium ion which distributes itself across the plasma membrane in accordance with the membrane potential (12). Furthermore, we have compared the effects of TRH to those induced by high extracellular K+ which is known to depolarize pituitary cells and enhance membrane permeability to Ca2+.

EXPERIMENTAL PROCEDURES

Cell Culture—GH3 cells (American Type Culture Collection) were grown in Ham's F-10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum (13). Sixteen to forty hours prior to an experiment, the cells were harvested with EDTA (0.02%) and incubated in Eagle's minimal essential medium for suspension culture supplemented with 15% horse and 2.5% fetal bovine serum (14). Cells for Ca2+ and 3HJTPMP' efflux experiments were incubated in the presence of Ca2+ (2 μCi/ml) also. Immediately prior to an experiment, the cells were harvested by centrifugation at 180 × g for 5 min and resuspended in a solution containing 135 mM NaCl, 4.5 mM KCl, 1.5 mM CaCl2, 1.3 mM MgCl2, 5.6 mM glucose, 1 g/liter of ovalbumin, and 10 mM Hepes (pH 7.4) (BSS).

Cell Perfusion—The perfusion system is a modification of the method described by Lowry and McMartin (15). Fifteen million cells were stirred with 1 ml of settled resin (Bio-Gel P-2, 200 to 400 mesh, Bio-Rad Laboratories) which had been swollen in BSS and placed in a 3-ml disposable syringe on top of a nylon mesh (20 μm). Two milliliters of additional resin were placed on top of the cell column and it was perfused with BSS (0.5 ml/min) at 26°C; the perfusate was collected in a fraction collector.

TJPMP' Distribution Measurements—Cells (1 × 10⁶/50 μl of BSS) were incubated at 26°C in the presence of 0.1 mM [3HJTPMP'] (360 Ci/mole, New England Nuclear) with constant stirring. At the indicated times, 50-μl portions were taken in duplicate, layered on top of 55 μl of silicone F-20 in 400-μl polyethylene tubes, and centrifuged at 8,000 × g for 60 s in a high speed centrifuge (Eppendorf model 2200) (16). The tubes were cut below the silicone layer and the cell pellets were counted. A zero time control was obtained by separating cells immediately after addition of [3HJTPMP'] to correct for adsorption of [3HJTPMP'; this value was subtracted from all experimental values.

Ca2+ and [3HJTPMP'] Efflux Studies—These were performed employing the perfusion system by measuring Ca2+ and [3HJTPMP'] radioactivity in the perfusate. For the Ca2+ efflux experiments, cells were incubated for 16 to 40 h in the presence of Ca2+ (2 μg/ml) as ionophores, are not specific (2), it has been not possible to demonstrate the pool(s) from which Ca2+ is mobilized by TRH. In fact, differentiation between an extracellular and intracellular pool(s) has not been made. If TRH were to induce an influx of extracellular Ca2+, this might occur either through a plasma membrane potential-dependent or independent permeability channel. In recent experiments in which intracellular microelectrode recordings were performed, Dufy et al. (9) demonstrated that the TRH-induced increase in the frequency of action potentials generated by prolactin-secreting pituitary cells occurred in the absence of any detectable change in the resting membrane potential. Since action potentials generated by these cells were reduced or abolished by a Ca2+ channel blocker and only slightly changed in Na+-free medium, it was concluded that they were generated by a Ca2+ mechanism. These data suggest that after TRH, Ca2+ may be mobilized across the plasma membrane in the absence of any change in membrane potential.
described above. In different experiments, stimuli were 100 nm [3H]-TRH (15 Ci/mmol, New England Nuclear), 1 μm unlabeled TRH (Beckman), or 50 mM K+, prepared by substituting KCl for NaCl in the BSS (high K+ BSS). For the experiments in which, in addition, [3H]TPMP+ efflux was measured, 7.5 × 10^6 cells were incubated with 45Ca2+ prior to stirring with the resin. For the [3H]TPMP+ efflux studies, the cells were perfused with BSS with 0.1 mM unlabeled TPMP+. 45Ca and 3H radioactivity were analyzed in a dual region liquid scintillation counting system (Tri-Carb 460C, Packard Instruments); counts in the 3H region were corrected for spillover of 45Ca.

Rat prolactin was measured in the perifusate by a double antibody radioimmunoassay (17) using reagents kindly supplied by the National Institute of Arthritis, Metabolism and Digestive Disease Pituatory Hormone Distribution Program and Dr. A. F. Parlow.

RESULTS AND DISCUSSION

The time course of the effect of TRH on 45Ca2+ efflux from GH3 cells is shown in Fig. 1. The cells in the column had accumulated 38,000 cpm of 45Ca106 cells during the preincubation period. There was a nearly constant rate of 45Ca+ efflux after 13 min of perfusion. A 0.6-min pulse (from 16.2 to 16.8 min) of [3H]TRH induced a dramatic increase in the efflux rate which persisted for at least 3.5 min. There was a delay of between 0.8 and 1.0 min between the appearance of the [3H]TRH and 45Ca2+ radioactivity peaks. This delay appears to be due to exclusion of [3H]TRH from the polyacrylamide beads in which the cells are supported since, when [3H]TRH and 45Ca2+ were injected simultaneously into the perfusion system in the absence of cells, a similar delay was observed. Therefore, no measurable lag period for the TRH effect on 45Ca2+ efflux was found.

The time course of TPMP+ uptake is shown in Fig. 2. When the proton conductor carbonyl cyanide m-chlorophenylhydrazone or high K+ was added, the accumulated TPMP+ was lost from the cells. These observations are consistent with the suggestion that TPMP+ is distributing across the GH3 plasma membrane in accordance with the membrane potential difference since these factors are known to depolarize cells. After approximately 90 min, in control incubations, cell-associated TPMP+ reached a plateau value of 0.80 nmol/106 cells. The intracellular volume of GH3 cells was measured using capillary tubes and found to be 1.4 ± 0.27 μl/106 cells (mean ± S.E.). Using this value, it was possible to calculate the ratio of intracellular to extracellular concentrations of TPMP+. Insertion of this ratio into the Nernst equation gave a value of ~50 ± 1.7 mV for the membrane potential of GH3 cells. This value is identical to that obtained by direct intracellular recording (~49 ± 0.27 mV) (9), thus, TPMP+ distribution appears to be a valid measure of membrane polarization in GH3 cells.

TRH had no effect on the accumulation of TPMP+ in these static incubations (Fig. 2). This was so if TRH and TPMP+ were added to GH3 cell suspensions simultaneously or if TRH was added after TPMP+ had reached its plateau value (18, 19). These data suggest that TRH has no effect on resting membrane polarization of GH3 cells and confirm the findings of Dufy et al. (9).

The effects of TRH and high K+ on 45Ca2+ efflux, [3H]-TPMP+ efflux, and prolactin release measured simultaneously are shown in Fig. 3. This system allows for simultaneous measurement of these parameters, thereby permitting analysis of the temporal sequence of these changes which may suggest a causal relationship. Because a homogeneous (cloned) population of cells was used in these studies, the changes observed are probably occurring in all cells. In agreement with the findings of other workers, TRH and high K+ caused an increase in the rate of efflux of 45Ca2+ (5) and stimulated release of prolactin (4). In this perfusion system, the amount of prolactin released by high K+ was greater than that released by 1 μM TRH. In contrast to the qualitatively similar effects of TRH and K+ on 45Ca2+ efflux and prolactin release, the effects on [3H]TPMP+ efflux were different. High K+ caused a dramatic increase in the rate of efflux of [3H]TPMP+ which was consistent with depolarization of the plasma membrane as observed in the static incubations. TRH had no detectable effect on [3H]TPMP+ efflux in this and several other perfusion systems. Therefore, no measurable lag period for the TRH effect on 45Ca2+ efflux was found.

FIG. 2. Uptake of TPMP+ by GH3 cells. GH3 cells (1 × 10^6 cells/50 μl) were incubated in BSS containing 0.1 μM [3H]TPMP+ (360 Ci/mmol) at 26°C with constant stirring (control, O). TRH (1 μM) was added to identical cell suspensions at zero time (C) or after 90 min of incubation (∇). After 40 min, equal portions of cells were centrifuged at 180 x g for 5 min and resuspended in BSS with 0.1 mM [3H]TPMP+ (control), BSS with 0.1 mM [3H]TPMP+ and 1 mM carbonyl cyanide m-chlorophenylhydrazone (CCCP, △), high K+ BSS with 0.1 mM [3H]TPMP+ (■) and incubated for an additional 20 min. At the times indicated, 50 μl portions were taken in duplicate and the cells were separated from the medium by centrifugation through a layer of silicone at 8,000 x g for 60 s. A zero time control value was subtracted from each point. Duplicate values varied by no more than 8%.

FIG. 1. Effect of TRH on 45Ca2+ efflux from GH3 cells. GH3 cells were incubated at 37°C in Eagle’s minimal essential medium for suspension culture supplemented with 15% horse and 2.5% fetal bovine sera and 45Ca2+ (2 μCi/ml). After 40 h, 15 × 10^6 cells were placed in a perfusion system and perfused with BSS (0.3 ml/min) at 36°C. After 16.2 min, 100 mM [3H]TRH was added to the perfusate solution for 0.6 min. The perfusion effluent was collected at 0.2-min intervals and analyzed for 45Ca and 3H radioactivity. Inset, [3H]TRH radioactivity on an expanded scale.
TRH Effect on Ca\(^{2+}\), Membrane Potential, and Prolactin Release

**Fig. 3. Effects of TRH and high K\(^+\) on \(^{45}\)Ca\(^{2+}\) efflux, \([\text{H}]\)-TPMP\(^{+}\) efflux, and prolactin release from GH\(_3\) cells.** GH\(_3\) cells were incubated with \(^{45}\)Ca\(^{2+}\) (2 \(\mu\)Ci/ml) for 16 h as described (see Fig. 1). Another portion of cells was incubated in BSS with 0.1 mm \([\text{H}]\)-TPMP\(^{+}\) (360 Ci/mmol) at 26\(^\circ\)C for 2 h. Equal numbers of cells (7.5 \(\times\) 10\(^5\)) prelabeled with \(^{45}\)Ca\(^{2+}\) or \([\text{H}]\)-TPMP\(^{+}\) were mixed, placed in the perfusion column, and perfused with BSS containing 0.1 mm unlabeled TPMP\(^{+}\) (0.5 ml/min) at 26\(^\circ\)C. After 28 min, 1 \(\mu\)M TRH was added to the perfusate for 1 min and after 41 min, the perfusate was changed to high K\(^+\) BSS with unlabeled TPMP\(^{+}\) for 1 min. The perfusion effluent was collected at 1-min intervals and analyzed for \(^{45}\)Ca and \([\text{H}]\) radioactivity and prolactin. The dashed line in the upper panel represents the limit of detectability of the assay.

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