Dual effects of thyrotrophin-releasing hormone (TRH) on K\(^+\) conductance in frog pituitary melanotrophs. TRH-induced \(\alpha\)-melanocyte-stimulating hormone release is not mediated through voltage-sensitive K\(^+\) channels

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ABSTRACT

Modulation of the activity of K\(^+\) channels by TRH and the possible involvement of this modulation in TRH-induced release of \(\alpha\)-MSH were studied in cultured frog melanotrophs, using patch-clamp and perfusion techniques. Pars intermedia cells were enzymatically dispersed and cultured in Leibovitz medium. In order to test the viability of cultured cells, the amount of \(\alpha\)-MSH released into the medium was measured by radioimmunoassay every day for 1 week of culture. The total amount of \(\alpha\)-MSH released during the first 4 days of culture was 8.6 times higher than the intracellular content of \(\alpha\)-MSH on day 1. Melanotrophs were identified by an indirect immunofluorescence technique using a specific antiserum to \(\alpha\)-MSH. Recordings obtained in whole-cell, cell-attached and excised patch-clamp configurations showed that TRH induced a transient polarization concomitant with an increase in the probability of opening of Ca\(^{2+}\)-activated K\(^+\) channels. This transient response was followed by a depolarization accompanied by an enhanced frequency of action potential discharge. TRH also induced a decrease in voltage-dependent K\(^+\) conductance. Application of tetraethylammonium, a K\(^+\) channel blocker, depolarized the cells and increased the basal secretory level without noticeable changes in TRH-evoked \(\alpha\)-MSH release.

These results demonstrate that the neuropeptide TRH both stimulates Ca\(^{2+}\)-sensitive K\(^+\) channels and inhibits voltage-dependent K\(^+\) current in pituitary melanotrophs. Our data indicate that TRH-induced secretion of \(\alpha\)-MSH is not a direct consequence of the lowering of K\(^+\) conductance. It thus appears that basal and TRH-induced \(\alpha\)-MSH release occur through distinct pathways; the spontaneous release of \(\alpha\)-MSH is probably linked to membrane potential, while modulation of the electrical activity is not directly involved in TRH-induced activation of the secretory process.

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INTRODUCTION

The tripeptide pyro-Glu-His-Pro-NH\(_2\) was originally isolated and characterized from the ovine brain for its ability to stimulate the secretion of thyrotrophin (TSH) (Burgus, Dunn, Desiderio & Guillemin, 1969) and was thus named thyrotrophin-releasing hormone (TRH). In mammals, TRH stimulates both TSH and prolactin secretion from anterior pituitary cells (Bowers, Friesen, Gudy & Folkers, 1971; Vale, Grant & Guillemin, 1973). It also triggers the release of prolactin and growth hormone from the GH\(_3\) cell lines derived from a rat anterior pituitary tumour (Tashjian, Yasumura, Levine et al. 1968; Gourdi, Tixier-Vidal, Morin et al. 1973). In amphibians, TRH stimulates prolactin secretion (Hall & Chadwick, 1984) but has no effect on TSH release (Taurog, Oliver, Eskin et al. 1974). In fish and amphibians, TRH also has the remarkable ability to stimulate the release of \(\alpha\)-melanocyte stimulating hormone (\(\alpha\)-MSH) from pituitary melanotrophs (Tonon, Leroux, Lebounglen et al. 1980a).

There is now clear evidence that TRH-induced stimulation of prolactin secretion by GH\(_3\) cells or rat anterior pituitary cells is associated with modifications of the electrical activity of the membrane of these cells. TRH induces a transient hyperpolarization and a long-lasting depolarization. This depolarization is responsible for an increased frequency of action potentials in spontaneously active cells, or the
appearance of firings in previously silent cells (Ozawa & Kimura, 1979).

We have recently shown that frog melanotrophs exhibit action potentials which are predominantly sodium spikes with a calcium component (Louiset, Cazin, Lamacz et al. 1988). In order to determine the possible involvement of the electrical activity of pituitary melanotrophs in stimulus–secretion coupling, we have investigated the effect of TRH on membrane potential, potassium conductance and secretory activity of frog pars intermedia cells. We report that, in agreement with studies conducted in normal (Rivier & Vale, 1974; Taraskevich & Douglas, 1977) and tumoral (Gourdji et al. 1973; Taraskevich & Douglas, 1980) mammalian pituitary glands, TRH modulates both the secretory and electrical activities of frog melanotrophs. TRH generates a transient outward calcium-activated potassium current, giving rise to a hyperpolarization, and produces a decrease in the voltage-dependent potassium current (IKv), causing a sustained depolarization. In parallel, we have demonstrated that blockade of K+ currents by tetraethylammonium (TEA), which depolarizes the cells, does not affect their secretory response to TRH.

MATERIALS AND METHODS

Cell cultures

Frogs (Rana ridibunda) were decapitated and the neurointermediate lobes of the pituitary were carefully separated under a dissecting microscope. The cells were enzymatically dispersed as described previously (Louiset et al. 1988). Briefly, eight lobes were incubated for 30 min at 26 °C in Leibovitz culture medium containing 0.5% (w/v) collagenase type IA, 1% (w/v) protease type IX, 0.1 mg kanamycine/ml and 1% (v/v) antibiotic–antimycotic solution. Leibovitz medium, collagenase and protease were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Kanamycine and the anti-biotic–antimycotic solution were purchased from Boehringer (Meylan, France). The dispersed cells were diluted to a density of 250 000 cells/ml in Leibovitz medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and antibiotics. Pars intermedia cells used for electrophysiological studies were routinely cultured in microwell Terasaki dishes (Consortium de Matériel pour Laboratoires, Nemours, France). For measurements of hormone secretion and immunohistological studies the cells were plated on Lab-Tek chambers (Poly Labo, Strasbourg, France) coated with gelatin and 1-polylysine. These chambers were incubated with a solution of autoclaved porcine skin gelatin (250 μg/ml) for 30 min, and the gelatin solution was then aspirated. For 1-polylysine coating, the polymer (10 μg/ml; Mw 70 000–150 000) was dissolved in 0.15 M sodium borate buffer (pH 8.4). After overnight incubation the dishes were rinsed three times with 0.1 M phosphate-buffered saline (PBS; pH 7.4). The cells were allowed to attach to the dishes for 24 h at 28 °C in a humidified atmosphere. The incubation medium was then replaced by fresh medium every day.

Quantification of hormone secretion

The amount of α-MSH released by cultured pars intermedia cells was measured every 24 h for 7 days. The cultured cells were rinsed and incubated with 200 μl Leibovitz culture medium for 1 h each day. The medium was then aspirated and centrifuged for 5 min at 10 000 g and the supernatant flash-frozen and stored at −20 °C. Serial dilutions of the cultured medium were prepared and the α-MSH concentration was radioimmunoassayed in duplicate in each sample. All dilution curves obtained with culture medium were parallel to the standard curve.

Perfusion of pituitary cells

The perfusion column was constructed according to Tonon, Leboulenger, Delarue et al. (1980b), the present method differing only in the use of enzymatically dispersed pituitary cells instead of whole neurointermediate lobes. Briefly, 50 000 freshly dissociated cells were gently mixed with 1 ml preswollen Bio-Gel P2 (Bio-Rad Laboratories, Richmond, CA, U.S.A.) and packed in a siliconized glass tube. The cells were perfused at a constant flow rate of 0.6 ml/min. The perfusion medium contained 112 mM NaCl, 2 mM KCl, 2 mM CaCl2, 15 mM Hepes and 11 mM glucose, and was supplemented with bovine serum albumin (0.3 g/l). The solution was gassed for 15 min with 95% O2:5% CO2 and the pH adjusted to 7.4 with NaOH. The temperature of the medium was maintained at 28 °C. The cells were allowed to stabilize for 30 min before the experimental manipulations commenced. The following substances were tested in the system: TEA, picrotoxin (Sigma Chemical Co.) and synthetic TRH (generous gift from Dr G. Kupryszewski, University of Gdansk, Poland). Test substances were dissolved directly in perfusion medium just before use. The effluent medium was collected as 7.5-min fractions during stabilization periods or 0.5- or 2.5-min fractions during test periods. The fractions were immediately chilled on ice and radioimmunoassayed for α-MSH, in duplicate, on the same day.
Radioimmunoassay

The α-MSH concentration was measured using a double-antibody radioimmunoassay method. The antiserum was raised against the carboxyl-terminal region of the α-MSH molecule (Vaudry, Tonon, Delarue et al. 1978). The sensitivity threshold of the assay was 5 pg/100 μl in perfusion medium and 40 pg/100 μl in Leibovitz culture medium supplemented with fetal calf serum and antibiotics. Perfusion experiments were carried out at least four times. The basal values were calculated as the mean α-MSH release in four consecutive fractions collected during the equilibration period.

Immunofluorescence identification of cultured cells

Immunocytochemical examination of cell cultures was carried out on 1- to 7-day-old cells. The culture medium was aspirated and the cells were rinsed three times with PBS before and after fixation for 10 min in methanol at −20 °C. All antisera were diluted in PBS supplemented with 1% (v/v) human serum albumin and 0.3% (v/v) Triton X-100. The cells were preincubated with normal goat serum (diluted 1:25) for 30 min at room temperature and rinsed three times with PBS. The cells were then incubated overnight in a moistened chamber at 4 °C with the antiserum to α-MSH (diluted 1:200). After rinsing with PBS, the cells were incubated for 1 h in a dark moistened chamber at 20 °C with fluorescein-isothiocyanate-conjugated anti-rabbit γ-globulin (Nordic Immunology, Tilburg, The Netherlands) diluted 1:60. The cells were counterstained for 3 min with Evan’s blue (diluted 1:10 000 in distilled water), mounted with PBS–glycerol (1:1, v/v) and examined under a Leitz Orthoplan fluorescence microscope. The specificity of the immunoreaction was controlled by using α-MSH antiserum preabsorbed overnight at 4 °C with 10 μM synthetic α-MSH.

Electrophysiological recordings

Electrophysiological studies were performed at room temperature (20–22 °C) on 2- to 7-day-old cells, using the patch-clamp technique (Hamill, Marty, Neher et al. 1981). The culture medium was replaced by an extracellular salt solution similar to that used for perfusion experiments. Variations from this standard solution are noted in the figure legends. The bath solution was continuously renewed by using a perfusion system. The electrodes were filled with the intracellular salt solution (100 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 10 mM EGTA and 10 mM Hepes; pH 7.4). The free calcium concentration was calculated to be 10 nM, assuming that the dissociation constant of the Ca²⁺-EGTA complex was 0.1 μM. All measurements were made with a patch-clamp amplifier (EPC 7 List Electronic, Eberstadt, F.R.G.) operated in current-clamp or voltage-clamp mode. In some experiments depolarizing pulses or ramps were given by an SMP Programmable Stimulator (Biologic, Echirôles, France). Synthetic TRH was applied for 3–30 s to the cell under study by pressure ejection from a glass pipette. A superfusion system was employed to deliver the TRH solution when longer periods of TRH administration were necessary.

The recordings were filtered at 3 kHz, stored on a video recorder (Sony, Clichy, France) and later replayed for analysis on a digital oscilloscope (Nicholt, Madison, WI, U.S.A.). The data acquisition was accomplished at 12-bit digital resolution. In whole-cell voltage-clamp experiments, leakage and capacitive currents were digitally subtracted.

RESULTS

Secretory activity

In order to test the viability of pituitary cells in culture, the secretion rate of α-MSH was monitored over 1 week (Fig. 1). The α-MSH content of 16 freshly dissociated neurointermediate lobes was 623.9 ± 13.8 ng (mean ± s.e.m.). After a 24-h attachment period, the rate of release of α-MSH was 55.2 ± 6.5 ng/100 μl per h (mean ± s.e.m. of nine independent experiments), the interexperiment variations depending on the number of attached cells. The secretory activity then decreased linearly to a minimum of 19.9 ± 10.1 ng α-MSH/100 μl per h on

![Figure 1. Release of α-MSH-like immunoreactivity (α-MSH-LI) by frog intermediate lobe cells grown in primary culture. The concentration of α-MSH-LI released in the culture medium was measured by radioimmunoassay. The data are expressed as ng α-MSH-LI/100 μl culture medium per h. Each value is the mean ± s.e.m. of nine independent determinations.](image-url)
day 4 of culture. Thereafter, the secretion rate increased slowly to reach 35.6 ± 14.0 ng α-MSH/100 μl per h on day 7 of culture. The total amount of α-MSH released during the first 4 days was 5300 ng/Petri dish. Histochemical controls of cultured pituitary cells were performed using the indirect immunofluorescence technique. All cultured cells exhibiting a spherical, bipolar or multipolar shape displayed strong immunoreactivity for α-MSH (Fig. 2a). The remaining unlabelled cells were identified as being fibroblasts. Preabsorption of the antiserum with synthetic α-MSH (1 μM) resulted in complete loss of immunoreaction (Fig. 2b). During recording experiments the melanotrophs could be easily identified on the basis of their typical shapes, as illustrated in Fig. 2c.

**Effect of TRH on secretory activity**

The perifusion technique was applied to determine the ability of TRH to modulate the secretory activity of acutely dispersed melanotrophs. Administration of a 10-min pulse of TRH (0.1 μM) induced a biphasic stimulation of α-MSH release (Fig. 3). During the first phase the secretion of α-MSH rapidly increased, reaching a maximum within 2 min (532% of the basal level). Despite continued TRH infusion, the secretion rate of α-MSH then decreased dramatically to about 180% of basal. In some experiments, however, the rapid decrease of α-MSH release was followed by a plateau (see Fig. 11).

**Effects of TRH on membrane potential and spontaneous activity**

The effect of TRH on the electrical properties of frog melanotrophs was studied in 26 cells in the whole-cell configuration. Among these, 15 (58%o) responded to TRH applications and 11 (42%o) did not show any apparent response. In 3/15 cells the response pattern was characterized by an initial slight hyperpolarization, varying in magnitude from 5 to 10 mV and persisting for 20–30 s (Fig. 4a). There-

![Figure 2](https://via.placeholder.com/150)

**Figure 2.** Immunofluorescence photomicrographs of frog intermediate lobe cells after 2 days of culture. (a) Intense labelling was observed in melanotrophs with a spherical, bipolar or multipolar shape, while fibroblasts (arrows) were not stained. (b) Control cells incubated with α-MSH antiserum preabsorbed with synthetic α-MSH (1 μM) were not stained. (c) Melanotrophs are shown under phase-contrast optics. Scale bar = 20 μm.

![Figure 3](https://via.placeholder.com/150)

**Figure 3.** Effect of TRH on secretion of α-MSH-like immunoreactivity (α-MSH-LI) by dispersed frog melanotrophs. After a 30-min equilibration period, TRH (0.1 μM) was infused for 10 min. The data represent the mean ± S.E.M. of eight independent experiments. The spontaneous level of release of α-MSH-LI (100%, basal level) was calculated as the mean secretion rate of α-MSH-LI during the equilibration period (four consecutive fractions; ○). The spontaneous secretion rate of α-MSH-LI was 336 ± 33 pg/50 000 cells per min.
Transient polarization induced by TRH and calcium-activated K+ channel

In order to determine the ionic mechanisms underlying the initial polarization caused by TRH, whole-cell recordings were carried out during depolarizing ramps. Because of its rapidity of achievement, the ramp protocol was preferred to the increasing pulse procedure for characterizing the major ionic currents involved in the transient response induced by TRH. Figure 5 shows a representative example of the effect of TRH observed in voltage-clamp mode, during a depolarizing ramp reaching 0 mV from a holding potential of −100 mV. In control conditions the slowly rising depolarization activated a net outward current at potentials higher than −30 mV, a potential value corresponding to the activation thresholds of the delayed rectifier and the calcium-activated K+ currents (Louiset et al. 1988). The current amplitude then increased for more positive potentials. Immediately after the onset of microejection of TRH, the activation threshold appeared to be lower (−45 mV instead of −30 mV) over a period of 20–30 s. For potentials more positive than −45 mV, the current amplitude was higher than that measured in control conditions. To determine whether the net outward current induced by TRH resulted from an increased outward K+ current or inward Cl− current, or whether it could be accounted for by a decreasing inward Na+ or Ca2+ current, we performed recordings in conditions where K+ channels were blocked by TEA. In all cells tested (n=4), superfusion of TEA (20 mM) provoked an almost complete disappearance of the outward current which normally appears at poten-

Figure 4. Typical recordings of membrane potential and discharge frequency in response to TRH application in three cultured frog melanotrophs. (a) TRH first triggered a weak and transient polarization, leading to a cessation of firing. This early phase of the response was followed by a slight depolarization with a concomitant increase in the rate of action potential firing. (b and c) Recordings are shown from two other cells which exhibited only a clear depolarization with simultaneous discharge of action potentials in response to TRH application. In a, b and c, TRH (0.5 μM) was applied by delivery pipette for periods of 30 or 10 s as indicated by the bars. Numbers on the left of each record correspond to the potential levels. Time calibration (in a) is the same for all three records.

Figure 5. Effect of TRH on outward K+ current in cultured frog melanotrophs. A net outward current (lower traces) was elicited by depolarizing voltage ramps (upper trace). The voltage varied from a predetermined potential of −100 mV to 0 mV in a linear manner, so as to depolarize the cell. After the control recording was obtained, TRH was administered to the cell from the delivery pipette for 30 s. The current trace was recorded 5 s after the onset of TRH administration. The recovery trace was achieved 10 s after the end of TRH ejection. When tetraethylammonium (TEA; 20 mM) was introduced into the extracellular solution, the net outward current was suppressed. The external medium contained 102 mM NaCl, 12 mM KCl, 2 mM CaCl2 and 15 mM Hepes (pH 7.4).
tials more positive than $-30$ mV. This clearly indicated that the outward current observed in this range of potentials was mainly carried by potassium ions. This observation also suggested that the increase in the net outward current induced by TRH may result from an increase in $K^+$ conductance. To test this hypothesis the effect of TRH on the properties of $K^+$ channels was examined.

As it was assumed that TRH might interact with a receptor–channel complex or activate a second messenger system which modifies the channel activity, this study was conducted on cell-attached ($n = 6$) and outside-out ($n = 3$) patches. The study in the cell-attached configuration was performed under conditions where the $K^+$ concentration (100 mM) in the pipette solution was assumed to be similar to the intracellular concentration of this ion. Figure 6 shows an example of single-channel recording where the activity consisted of short openings of weak outward currents at the resting potential. When the patch was depolarized, the channel displayed longer and more frequent openings with few closure flickers (Fig. 6a). No open events were detected at hyperpolarizing potentials. The steady-state current-to-voltage relationship was linear between 0 and $+20$ mV and exhibited rectifying properties at more positive depolarizing potentials (Fig. 6b).

The value of the channel conductance measured at 0 mV was 230 pS. The outward current reversed at a potential close to 0 mV, a potential value which was supposed to be the potassium equilibrium potential in the present experiment. Consequently, this observation strongly suggests that this type of channel, characterized by a large conductance, was selective for $K^+$. Application of TRH in the bathing solution increased the open probability without changing the current intensity (Fig. 6c).

To test whether there was a possible direct effect of TRH on large-conductance $K^+$ channels, experiments were performed on outside-out patches. The external $K^+$ concentration was lowered to its physiological level (2 mM) by substitution with Na+. Under these conditions, the current–voltage relationship became non-linear as illustrated in Fig. 7. Moreover, in agreement with data obtained in smooth muscle cells (Benham, Bolton, Lang & Takewaki, 1986), the conductance was remarkably lower when measured in the inside-out configuration (where the internal concentration of $K^+$ was 2 mM) than that measured in the cell-attached conditions (with an internal $K^+$ concentration of 100 mM). At hyperpolarizing potentials, the opening events were so rare that the reversal current was never observed. Nevertheless, the current–voltage curves became asymptotic to the abscissa, which in-

**Figure 6.** Effect of TRH on single-channel outward $K^+$ current recorded from a cell-attached membrane patch in cultured frog melanotrophs. (a) Current records are shown at various holding potentials. (b) The corresponding I–V relationship gave a conductance of 230 pS at the resting potential. (c) TRH was applied to non-patched cell membrane over a period of 2 min. Records after control show an increase in the probability of channel opening in response to application of TRH (0.5 μM) for 5 s and 1.5 min. Recovery was observed during rinsing with extracellular solution. An expansion of a part of each trace is shown below.

**Figure 7.** Large-conductance $K^+$ channel recorded from an outside-out patch in control conditions (upper trace) and after 15 s of TRH (1 μM) application (lower trace) in a cultured frog melanotroph. The holding potential was +30 mV. Note the lack of change in the opening probability during TRH treatment. The I–V curve shown below corresponds to the same patch in normal saline.

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dictated that this type of channel was selective for $K^+$ over $Na^+$. Administration of TRH onto the extracellular surface of the excised patch did not modify the open channel probability, neither did it alter the current intensity (Fig. 7).

Large-conductance $K^+$ channels were also recorded in inside-out patches with different internal calcium concentrations. As illustrated in Fig. 8a the open probability dramatically increased when the excised patch was bathed in solutions containing calcium at concentrations ranging from 1 to 10 $\mu$M. When internal and external $K^+$ concentrations were equal, the reversal potential was 0 mV, as would be expected for a $K^+$-selective channel (Fig. 8b and c). On the basis of all these data, the large-conductance $K^+$ channel activated by TRH was thus identified as a calcium-activated potassium channel.

**Delayed depolarization induced by TRH and voltage-dependent $K^+$ current**

The effect of TRH on the voltage-dependent $K^+$ current was studied in cells bathed in a medium where sodium and calcium were replaced by choline and cobalt respectively. In these conditions, the slowly inactivating outward current ($I_{Kv}$) elicited by a depolarizing pulse is considered as passing mainly through voltage-dependent potassium channels (Louiset et al. 1988). The amplitude of the peak outward current was measured before and during prolonged TRH administration (1–3 min). The inset of Fig. 9a shows an example of an outward current evoked during a constant test pulse in control conditions, and after 1 min of TRH application, and during rinsing with extracellular solution. TRH reversibly decreased the amplitude of $I_{Kv}$. The average time-course ($n = 9$) of the peak current amplitude is illustrated in Fig. 9a. Evidence that TRH depressed $I_{Kv}$ was also provided by the current–voltage curves obtained in control conditions and during TRH administration (Fig. 9b). For each test potential, the peak current amplitude decreased by a constant rate of about 35% during TRH infusion.

**$K^+$ currents and stimulus–secretion coupling**

To examine the possible role of $K^+$ currents in stimulus–secretion coupling, we studied the effect of TEA on membrane potential and on basal and TRH-stimulated secretion of $\alpha$-MSH. The effect of TEA on membrane potential was studied in whole-cell recordings ($n = 7$) under current-clamp condi-

![Figure 8](image-url)  
**Figure 8.** Calcium sensitivity of the large-conductance $K^+$ channels in cultured frog melanotrophs. (a) An inside-out patch was exposed to a symmetrical $K^+$ concentration (100 mM) and the membrane potential was set at +20 mV. As the internal free $Ca^{2+}$ concentration increased from 0.01 to 10 $\mu$M, the channel opened more frequently and for longer periods of time. (b) Current records are shown at various potentials in a high calcium concentration (10 $\mu$M). The current reversed at 0 mV, corresponding to the potassium equilibrium potential. (c) The I–V curve gave a conductance of 286 pS. The bathing solutions contained 100 mM KCl, 1 mM MgCl$_2$ and 10 mM Hepes (pH 7.4). The free $Ca^{2+}$ concentrations of 0.01, 1 and 10 $\mu$M were buffered with 11, 1.1 and 0.2 mM EGTA respectively.
tions. As illustrated on Fig. 10, in normal saline a pulse of depolarizing current activated an action potential. We have previously demonstrated the sodium and calcium dependency of the action poten-

tials in frog melanotrophs (Louiset et al. 1988). The addition of TEA in the bathing solution depolarized the cell from $-46$ to $-28$ mV. During the pulse of depolarizing current, the rectification of the membrane potential disappeared and a strong depolarization occurred. To investigate the involvement of K$^+$ currents in TRH-induced stimulation of α-MSH secon-
dition, TEA was administered to perifused pituitary melanotrophs. As shown in Fig. 11, TEA induced a significant ($P<0.05$; Student’s t-test) enhancement of basal α-MSH secretion and caused a weak transient decrease of α-MSH release at the onset of TRH administration. This inhibitory effect occurred within 1 min of TRH application and was followed by a peak of secretion. Despite this transient inhibition, the amount of α-MSH released was not significantly modified: TRH-induced α-MSH secretion was $732 \pm 124$ and $540 \pm 179$ ng/15 min in the absence and presence of TEA respectively.

To further our understanding of the ionic mecha-
nisms involved in the inhibitory phase of TRH-induced secretion observed in the presence of TEA, we tested the possible contribution of chloride ions, which are known to produce hyperpolarization by entering the cell. Perfusion with picrotoxin (0.1 mM), a chloride channel blocker, diminished basal α-MSH secretion (Fig. 11c) but did not affect TRH-induced secretion. As shown in Fig. 11d, the

\[
\begin{align*}
\text{TRH (10 nM)} & \\
0 & 50 100 150 200 250 300 \\
\text{IKV (\%)} & \\
\text{Control} & \\
\text{TRH} & \\
\text{Recovery} & \\
50 \text{ pA} & \\
50 \text{ ms} & \\
\text{Time (s)} & \\
\end{align*}
\]

Figure 9. Effect of prolonged TRH application on outward voltage-dependent K$^+$ current (IKv) in cultured frog melanotrophs. (a) The inset shows outward current records obtained before, during and after TRH (0.5 μM) superfusion. The currents were monitored by voltage steps from $-60$ mV to $+20$ mV, repetitively applied at 10-s intervals. The curve represents the mean time-course (±S.E.M.) of the effect of TRH on IKV in nine cells. The current was measured at its peak amplitude and expressed as a percentage of the mean IKV recorded before TRH administration. (b) I–V relationships in normal saline or during TRH treatment; note that the conductance became lower under TRH. The external solution contained 112 mM choline chloride, 2 mM CoCl$_2$, 2 mM KCl and 15 mM Hepes (pH 7.4).

\[
\begin{align*}
\text{Control} & \\
\text{TEA} & \\
\text{TEA} & \\
\text{[120 pA} & \text{200 ms]} & \\
\text{46} & \text{28} & \text{29} & \\
\text{50 mV} & \\
\end{align*}
\]

Figure 10. Effect of tetraethylammonium (TEA) on the resting potential and on the responses evoked by depolarizing (left and middle) and hyperpolarizing (right) constant current pulses in a cultured frog melanotroph. In normal saline the resting potential of the cell was $-46$ mV. The evoked response consisted of an action potential. In the TEA-containing solution (20 mM) the cell spontaneously depolarized to $-28$ mV. The responses observed during depolarizing and hyperpolarizing pulses reflected a change in the rectification phenomenon.

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transient inhibition which characterized the TRH response pattern observed in the presence of TEA was completely abolished when both TEA and picrotoxin were administered to the cells.

DISCUSSION

In the present study, cultured pars intermedia cells were used to investigate the effect of TRH on the electrophysiological activity of frog pituitary melanotrophs. Initially, the rate of hormone secretion from cultured pituitary cells was assessed in order to verify the feasibility and practicality of our cell culture procedure. Not surprisingly, the amount of immunoreactive α-MSH released by the cells decreased during the first 4 days of culture. In vivo, the frog pars intermedia is under tonic inhibitory control (Tonon, Leroux, Stoeckel et al. 1983) and massive release of hormonal peptide is expected to occur when these cells are disconnected from the influence of hypothalamic regulatory factors. The total amount of α-MSH released during the first 4 days was 5300 ng/Petri dish, which approximately corresponds to 8.5 times the intracellular content of α-MSH on day 1 of culture. This observation shows that cultured cells actively synthesize and release α-MSH-like peptides. The fact that the basal secretory rate slightly increased after day 5 of culture also indicates that melanotrophic cells maintain differentiated functions in our culture conditions.

The main objective of this research was to examine the involvement of K⁺ currents in the process of hormone release from pituitary melanotrophs. So far, most investigations undertaken to understand stimulus–secretion coupling in pituitary cells have
been conducted on tumoral cells, such as \textit{GH}3 cells (Ozawa & Kimura, 1979) or human adenoma cells (Israël, Jaquet & Vincent, 1985) which, in several respects, may not exhibit the same characteristics as normal endocrine cells. Only a few studies have been performed using normal pituitary cells, such as rat somatotrophs (Israël, Denef & Vincent, 1983) and bovine lactotrophs (Ingram, Bicknell & Mason, 1986) and porcine melanotrophs (Demeneix, Taleb, Loeffler & Feltz, 1986). Our experiments on freshly dispersed cells provide evidence for the existence of TRH receptors on the membrane of frog melanotrophs. This was previously suggested from perfusion studies carried out on neurointermediate lobes (Tonon \textit{et al.} 1983). The secretion rate of \(\alpha\)-MSH displayed two phases during a 2-h administration of TRH to the neurointermediate lobe. The second phase may reflect a desensitization of the TRH membrane receptor (Lamacz, Tonon, Danger \textit{et al.} 1987).

In the present study, using primary cultures of frog melanotrophs, we have demonstrated that TRH is a strong modulator of electrical activity. In a few cells this response was characterized by a slight and transient polarization, followed by a depolarization with a period of enhanced action potential activity. More frequently, the melanotrophs elicited only a depolarization, giving rise to spike discharges. The biphasic effect of TRH on membrane potential has already been reported from intracellular recordings in both tumoral (Ozawa & Kimura, 1979) and normal (Ingram \textit{et al.} 1986) pituitary cells. Such a biphasic response has also been observed from whole-cell recordings, in spite of the dialysis phenomena occurring in this patch-clamp configuration (Dubinsky & Oxford, 1985). Comparing classical intracellular with whole-cell current-clamp recordings, Dufy, Jaken & Barker (1987) concluded that the voltage response to TRH requires the presence of cytoplasmic factors such as protein kinase C.

The biphasic modification of membrane potential induced by TRH can be temporally correlated with changes in membrane ionic currents. We have observed that TRH initially increases an outward \(K^+\) current, giving rise to hyperpolarization. The single-channel recordings, in both cell-attached and excised patch configurations, demonstrated that TRH increases the open probability of \(Ca^{2+}\)-activated \(K^+\) channels via intracellular messengers. Calcium, released from intracellular stores, can be regarded as a putative intracellular messenger, since the activity of the large-conductance \(K^+\) channel was dependent upon the cytoplasmic \(Ca^{2+}\) concentration. This hypothesis is supported by the observation of Ritchie (1987) who showed that TRH simultaneously augments the intracellular \(Ca^{2+}\) concentration and increases the outward \(Ca^{2+}\)-activated \(K^+\) current. The calcium sensitivity of \(Ca^{2+}\)-activated \(K^+\) channels was studied using our inside-out patches. The open probability of \(Ca^{2+}\)-activated \(K^+\) channels was strongly increased in a range of calcium concentrations from 1 to 10 \(\mu\)M. When the calcium level on the cytoplasmic face was lower than 0.1 \(\mu\)M, \(Ca^{2+}\)-dependent \(K^+\) channels were not activated, whatever the holding potential values (not shown). The results are quite similar to those described in \textit{GH}3 cells by Lang & Richie (1987). It has also been shown from microfluorimetric recordings, however, that the intracellular calcium concentration can reach 0.1–1 \(\mu\)M in the presence of TRH (Geras-Raaka & Gershengorn, 1987; Ritchie, 1987). It thus appears that the increase in the internal concentration of calcium induced by TRH may not entirely account for the activation of \(Ca^{2+}\)-sensitive \(K^+\) channels. Other factors modulating the gating of these channels have consequently to be considered. For example, as found in a variety of other cell types, \(Ca^{2+}\)-activated \(K^+\) channels are also sensitive to the intracellular proton concentration (Christensen & Zeuthen, 1987) or to cAMP-dependent protein kinase (Ewald, Williams & Levitan, 1985).

Our data, together with previous reports on tumoral pituitary cells (Kaczorowski, Vandlen, Katz & Reuben, 1983; Dubinsky & Oxford, 1985), indicate that the delayed depolarization induced by TRH is probably linked to the long-lasting decrease of \(IK_V\). In fact, both TEA and TRH depolarized the cells and decreased \(IK_V\). Kaczorowski \textit{et al.} (1983) have suggested that TRH and TEA affect voltage-dependent \(K^+\) conductance by different mechanisms. TEA has a direct blocking effect on \(K^+\) channels (Armstrong, 1971) whereas TRH probably acts through a second messenger system. Using \textit{GH}3 cells, Dufy \textit{et al.} (1987) have demonstrated that diacylglycerol and protein kinase C are required for TRH-induced excitation. Moreover, in contrast to the early transient polarization, the delayed depolarization was not affected by any dialysis process. This would indicate that the effect of TRH is mediated by messenger(s) present within the plasma membrane. This conclusion is partially supported by the fact that diacylglycerol is included in the membrane and protein kinase C can generally be found in both cytoplastic and membrane preparations (for review see Kaczmarek, 1987).

The results of our parallel studies on the electrical and secretory activities strongly suggest that basal and TRH-induced release of \(\alpha\)-MSH may occur by two distinct pathways. Coupling is conceivable between the membrane potential and basal secretion. In fact, depolarization and enhanced release were observed when \(K^+\) channels were blocked by TEA.
Similar findings in rat and mouse melanotrophs have already been reported (Tomiko, Taraskevich & Douglas, 1984).

The early transient polarization results from the activation of an outward K\(^+\) current passing through calcium-activated K\(^+\) channels. This current, which is reminiscent of the increase in intracellular calcium concentration observed in GH\(_3\) cells (Gershengorn & Thaw, 1985; Ritchie, 1987) gives rise to the peak of TRH-induced α-MSH release. The delayed depolarization, which is a direct consequence of the decrease in IK\(_V\) (Dubinsky & Oxford, 1985), may induce calcium entry through voltage-activated and dihydropyridine-sensitive channels (Gershengorn & Thaw, 1985). As demonstrated in the present study, however, TRH-induced α-MSH secretion was not severely affected by blockade of K\(^+\) channels. The only change observed in the presence of TEA was a transient inhibition of α-MSH release which occurred during the first minute. This inhibitory phase, which was blocked by picROTOXIN, could be accounted for by an influx of chloride ions. The occurrence of this chloride current was probably related to the particular experimental conditions which placed the cells in a depolarized state. From these data, we conclude that TRH-induced α-MSH secretion is not a direct consequence of the lowering of IK\(_V\) elicited by TRH. In other words, the voltage-activated events are not essential, as previously proposed (Ozawa & Kimura, 1979), in the process of α-MSH release controlled by TRH. This concept is supported by the fact that neither dihydropyridines (Lamacz, Tonon, Guy et al. 1988) nor tetrodotoxin (data not shown), which are known to act on Ca\(^{2+}\) and Na\(^+\) channels respectively, can affect TRH-evoked α-MSH release. In very much the same way, the lack of coupling between TRH-induced electrical and secretory activities has recently been documented in normal bovine lactotrophs (Cobbett, Ingram & Mason, 1987).

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