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Characterization of an M-Like Current Modulated by Thyrotropin-Releasing Hormone in Normal Rat Lactotrophs

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In rat pituitary lactotrophs, a component of thyrotropin-releasing hormone (TRH)-induced prolactin secretion is dependent on extracellular calcium and is associated with an increase in action-potential firing. The mechanism underlying the TRH-induced increase in firing frequency was investigated using the perforated-patch variation of the whole-cell patch-clamp technique. TRH was found to inhibit a voltage-dependent, noninactivating K^+ current that was similar to M-currents originally identified in neurons. The TRH-modulated M-like current started to activate at ~−60 mV and had a V_0 of −28 mV and thus would be active under the normal resting potentials of lactotrophs (−35 to −45 mV). Exponential fits to deactivating tail currents revealed a fast and a slow component. The deactivation rates of the M-like current and the ratio of the fast to the slow component of deactivation were found to increase with more hyperpolarized potentials. In addition, increasing the duration of command potentials led to the conversion of the fast component to the slow component of deactivation. The M-like current in lactotrophs was partially sensitive to 4-aminopyridine and tetraethylammonium. TRH inhibition of this current was associated with acceleration of deactivation rates and a depolarizing shift in the voltage of activation (V_0 = −17 mV). The effect of TRH on the M-like current was lost in whole-cell voltage-clamp conditions, suggesting the requirement of an important cytosolic component that mediates the effect of TRH.

Key words: lactotroph; thyrotropin-releasing hormone; membrane potentials; voltage-dependent K^+ channel; GH_3 cells; M-current

Thyrotropin-releasing hormone (TRH) stimulates prolactin release from cells isolated from the rat pituitary gland (Vale et al., 1973) and from GH_3 cells, a rat prolactin and growth hormone-secreting tumor cell line (Tashjian et al., 1971). Studies in GH_3 cells have led to much of our understanding of the underlying mechanism of action of TRH. TRH induces a biphasic increase in secretion of prolactin by activation of the phosphoinositide turnover pathway (for review, see Gershengorn, 1986). The rapid first phase of secretion is attributable to the release of calcium from inositol trisphosphate-sensitive intracellular stores, whereas the slower second phase of secretion is dependent on the influx of calcium from extracellular sources (Albert and Tashjian, 1984; Martin and Kowalchyk, 1984; Aizawa and Hinkle, 1985; Gershengorn and Thaw, 1985). This biphasic effect on secretion and intracellular calcium levels is parallel to a biphasic response on the membrane-potential behavior (for review, see Ozawa and Sand, 1986). The first phase is a rapid hyperpolarization caused by activation of calcium-dependent potassium channels (Ritchie, 1987; Mollard et al., 1988). The second phase consists of slight depolarization associated with an increase in action-potential frequency (Ozawa and Sand, 1986). It has been suggested that the second phase is attributable to blocking of a voltage-dependent outward K^+ current (Dubinsky and Oxford, 1985); however, others have found no effect on voltage-dependent outward K^+ currents (Bauer et al., 1990; Simasko, 1991b). Alternatively, the increased excitability of the second phase has been associated with a decrease in an inwardly rectifying K^+ current (Bauer et al., 1990; Barros et al., 1992). Because this inwardly rectifying K^+ current is active at the resting membrane potential of the cell, it would be an ideal target for increasing the firing frequency of these cells.

Although in many respects the actions of TRH are similar to the effect produced in GH_3 cells, their actions on primary cultures of lactotrophs show some differences. In rat lactotrophs, TRH produced a biphasic response of calcium increase similar to that in the GH_3 cells (Malgaroli et al., 1987; Winiger et al., 1987; Law et al., 1988). In bovine lactotrophs, however, the plateau phase of calcium increase required pretreatment with forskolin (Akerman et al., 1991). The biphasic effects of TRH on membrane-potential behavior has also been observed in bovine lactotrophs (Ingram et al., 1986). Unlike that in GH_3 cells, in rat lactotrophs the initial hyperpolarization is attributable to the activation of calcium-dependent potassium and chloride conductances (Sartor et al., 1990, 1992).

Because the existence of an inwardly rectifying K^+ current has not been reported previously in primary cultures of lactotrophs, we investigated whether this current component existed in these cells and was modulated by TRH. Our results confirm that TRH does indeed modulate a K^+ current active at rest in lactotrophs, but further characterization of this current revealed that rather than having inwardly rectifying properties, its behavior is similar to the M-current described previously in neurons (Brown, 1988a).

MATERIALS AND METHODS

Cell culture. Rat pituitary lactotrophs were isolated by previous methods (Mason and Ingram, 1986; Sartor et al., 1990) with slight modifications. Anterior pituitaries from random cycling female Sprague–Dawley rats were separated from the posterior and intermediate lobes and diced into 1 mm cubes. The pituitary fragments were washed once in $Ca^{2+}/Mg^{2+}/HCO_3^-$-free Earle's balanced salt solution (CMF-EBSS) with 20 mM

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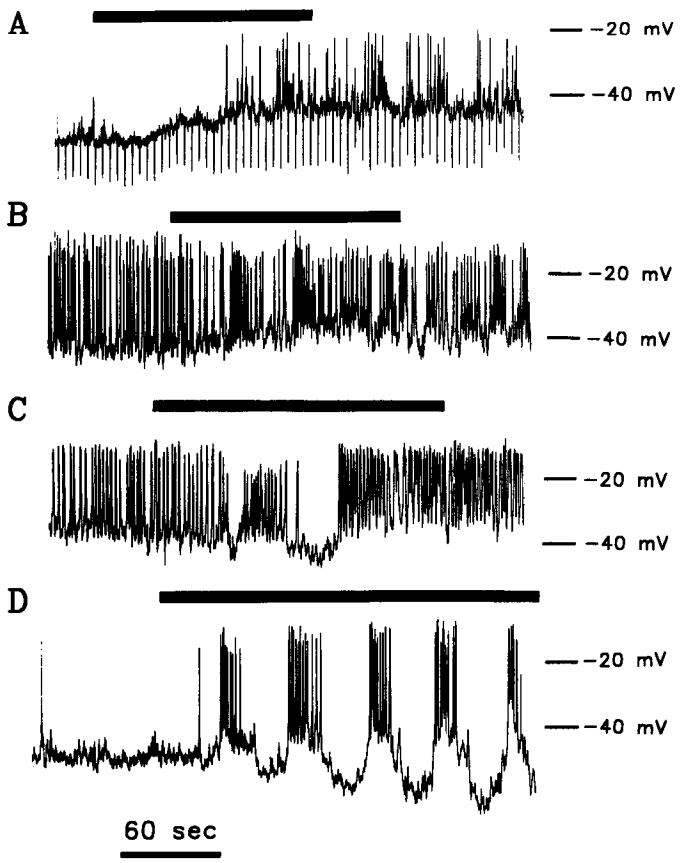

Figure 1. TRH depolarizes the basal membrane potential of lactotrophs and increases firing frequency. Illustrated are spontaneous action potentials of lactotrophs recorded using the perforated-patch variation of the whole-cell patch-clamp technique in standard physiological bath conditions. The *dark bars* above the traces (*A–D*) represent the duration of exposure to TRH at a concentration of 1 μM. The *time bar* shown at the *bottom* is the same for all traces. The potentials on the *right* indicate the voltage scale for each of the illustrated traces.

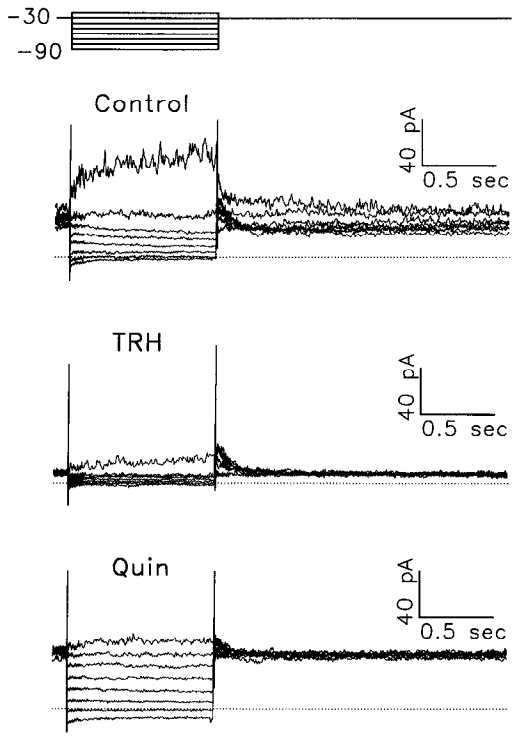

Figure 2. TRH inhibits an outward current active under normal resting potentials of lactotrophs. The *top trace* illustrates the voltage protocol (holding potential −30 mV, command pulses from −20 to −90 mV). The three sets of traces labeled *Control*, *TRH*, and *Quin* represent the current traces under control conditions and after exposure to 1 μM TRH and then to 10 nM quinpirole, respectively. The *dotted lines* in the three traces represent zero current. The *current* and *time scales* are illustrated to the *right* of each of the three sets of traces. These traces are not corrected for leak currents.

HEPES, pH 7.3, 2.8 mM glucose, and 1 mg/ml bovine serum albumin. The pituitary fragments were then incubated with 0.2% collagenase (in CMF-EBSS for 1 hr at 37°C), followed by a 10 min incubation in 0.01% DNase with 0.2% collagenase, followed by a final 10 min incubation in CMF-EBSS. The tissue fragments were then centrifuged and resuspended twice in CMF-EBSS, and a single-cell suspension was obtained after gentle disruption using a 2 ml pipette. Finally, cells were filtered through a 30 μm filter to remove any undispersed tissue pieces.

Lactotroph enrichment. Lactotrophs were enriched from the cell suspension (prepared as described above) by a discontinuous percoll gradient (60, 50, and 35% percoll, pH 7.3; osmolality ~300 mOsm) as described previously (Burris and Freeman, 1993), with minor modifications. The cells were mixed with an equal volume of 60% percoll, and 1.5 ml of this suspension (~5–7 × 10^6 cells) was placed in a 15 ml centrifuge tube. By using a syringe and a 10-cm-long 15 gauge needle, 2 ml fractions of each percoll solution were introduced underneath the cell suspension in the order of increasing density. The tube was then centrifuged at 450 × *g* for 20 min. The cells formed layers at the 35/50 and 50/60 interfaces. Each layer was recovered using a Pasteur pipette. The cells were counted and seeded on poly-L-lysine-coated glass coverslips in Ham's F-10 media containing 15% horse serum and 3% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2/95% air. Enzymes, chemicals, and percoll were obtained from Sigma (St. Louis, MO), and cell culture reagents were from Gibco (Grand Island, NY).

Immunocytochemistry. After 1 d in culture, cells were fixed at room temperature using 4% paraformaldehyde in PBS for 1 hr before washing with PBS. The coverslips were incubated overnight with a 1:16,000 dilution of anti-rat prolactin antibody (NIDDK-anti-rPRL-IC-5 rabbit). Antibody binding was visualized using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Cells positively stained for prolactin comprised 65–70% of cells isolated from the 35/50 interface and ~20% of cells from the 50/60 interface after percoll density gradient centrifugation (n = 7). Cells isolated from the 35/50 interface were used in all experiments in this study.

GH_3 cell culture. GH_3 cells were obtained from American Type Culture Collection (Rockville, MD) and were grown as described previously (Tashjian, 1979). A stock line was maintained by growing cells in 50 ml plastic culture flasks in 5 ml Ham's F-10 media supplemented with 15% horse serum and 2.5% fetal calf serum. The cells were incubated in a humidified atmosphere with 95% O_2 and 5% CO_2. Cells were subcultured once a week (split 1:3). At each pass, cells required for electrophysiological experiments were plated on glass coverslips. Experiments were performed on these cells between 4 and 10 d after plating. Cells were frozen in 10% glycerol in the above culture medium, and new stock lines were started once every 10–15 weeks. All cell culture media and supplements were from Gibco.

Electrophysiological measurements. All experiments were performed using the perforated-patch variation of the standard whole-cell patch-clamp technique (Horn and Marty, 1988) at room temperature, except where indicated. Electrophysiological recordings were performed as described earlier (Simasko, 1991b). Patch-clamp electrodes had tip resistances of 3–5 MΩ. Access resistances of <50 MΩ were generally obtained within 10–20 min after seal formation. In some cells, series resistance was compensated up to 50%; however, greater compensation led to oscillations. Although this results in some voltage-clamp errors in traces with large currents (typically during large tail currents), this error does not significantly alter the estimates of activation voltages because current levels at these potentials were small. The cells had input resistances between 5 and 10 GΩ. Cell input resistance was calculated by analysis of current responses to a series of voltage steps between −90 and −70 mV.

The standard pipette recording solution consisted of (in mM): 12 KCl, 59 K_2SO_4, 4 $MgCl_2$, 10 NaCl, and 10 HEPES, pH 7.4, with Tris. The

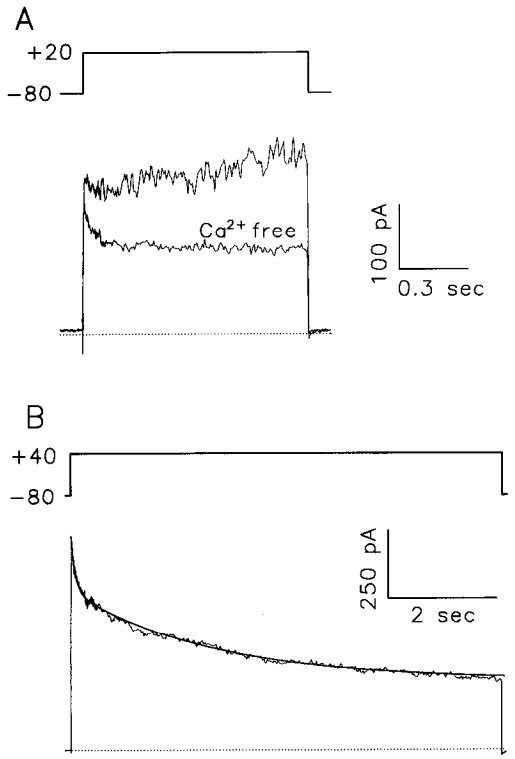

Figure 3. Outward K^+ currents observed in a standard physiological bath. *A*, The *top trace* illustrates the voltage protocol. The current trace labeled *Ca^{2+} free* is under conditions in which calcium in the standard bath is replaced with Mg^{2+}. The *dotted line* represents zero current. *B*, Inactivation of outward K^+ currents observed after 8 sec voltage pulse in Ca^{2+}-free bath. The *top trace* is the voltage protocol. The *thin wavy line* is the actual current trace, whereas the *dark superimposed line* is the inactivation fit to the current trace. The time constants are $\tau_1 = 82$ msec and $\tau_2 = 2.6$ sec. The *dotted line* represents zero current.

standard bath consisted of (in mM): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 6 glucose, and 10 HEPES, pH 7.4, with Tris. The characterization of the current modulated by TRH was performed mainly in a high K^+, Ca^{2+}-free solution (Hi K Ca-free). This solution consisted of (in mM): 130 KCl, 10 NaCl, 3 $MgCl_2$, 6 glucose, and 10 HEPES, pH 7.4, with Tris. In experiments in which lower KCl concentrations were used, KCl was replaced with equimolar NaCl. Solutions containing tetraethylammonium (TEA) or 4-aminopyridine (4-AP) were titrated to pH 7.4 after the addition of a blocker. TRH was obtained from Bachem (Torrance, CA). Quinpirole hydrochloride (LY171555) was from Research Biochemicals International (Natick, MA). All other chemicals were obtained from Sigma.

Results from current-clamp experiments were stored on VCR tape and analyzed by direct visualization of traces recorded onto a chart recorder. Voltage-clamp experiments were controlled and analyzed using a FASTLAB software package from INDEC Systems (Sunnyvale, CA). Leak currents were estimated by averaging the currents after three consecutive pulses to −90, −80, and −70 mV under control situations and after exposure to a pharmacological agent. Leak correction was performed on all traces illustrated, except where indicated. Time constants of deactivation were determined by exponential fits to deactivation of tail-current components (after appropriate leak correction). Theoretical fits to the data were evaluated with a nonlinear least-squares routine that used a modified Newton-Raphson iteration procedure (TKSolver software, Universal Technical Systems, Rockford, IL).

Statistics. All values reported are averages ± SE. Two-tailed paired *t* tests on amplitudes and rate constants of deactivating tail currents were used to determine whether an experimental condition caused a statistically significant change ($p < 0.05$).

RESULTS

Effects of TRH on spontaneous calcium-dependent action potentials

Current-clamp experiments were performed on lactotrophs under standard physiological bath conditions. Lactotrophs exhibited spontaneous action potentials that were not sensitive to tetrodotoxin, similar to those reported in earlier studies (Chen et al., 1987; Israel et al., 1987). In current-clamp experiments, 90% of the cells challenged with the dopamine agonist quinpirole (10 nM) had an immediate hyperpolarizing response (25/27, data not shown). This responsiveness to a dopamine agonist confirms that a large majority of the cells used in this study were indeed lactotrophs.

Illustrated in Figure 1 are typical examples of the effect of TRH on spontaneous action potentials in lactotrophs. The lactotrophs had a range of resting membrane potentials from −33 mV to −58 mV, the average resting or baseline membrane potential being −45.4 ± 1.5 mV ($n = 20$). TRH had no remarkable effect on the membrane-potential behavior of 5 of the 20 cells challenged with TRH. In 13 of the 15 cells that responded to TRH, a slight depolarization of the baseline membrane potential was observed (Fig. 1*A–C*) (+6.6 ± 0.6 mV, $p < 0.001$). We observed a transient hyperpolarization, however, after exposure to TRH in only 4 of 13 cells (−9.0 ± 0.6 mV). In cells in which spikes were clearly discernible, TRH increased the frequency of firing by 9.4 ± 1.7 spikes/min ($n = 11$; $p < 0.001$).

TRH was also capable of inducing the development of spiking activity in silent cells (Fig. 1*A*). In this recording, the development of spiking was associated with a decrease in membrane conductance, as seen by the increase in the size of the voltage response to a hyperpolarizing current injection. In two cells, we observed that TRH produced an oscillatory pattern of spiking behavior that was characterized by depolarization to a plateau with superimposed spikes on top of this plateau, followed by pronounced afterhyperpolarizations (Fig. 1*D*).

TRH inhibits an outward current, which is active at resting potentials of lactotrophs

When lactotrophs were voltage-clamped to a potential of −30 mV, they displayed an outward current (Fig. 2, *Control*). When the cells were held at −30 mV (hold time, 20 sec) and then pulsed to a series of potentials from −20 to −90 mV in 10 mV increments, an outward current present at −30 mV relaxed slowly as the cells were pulsed to more hyperpolarizing voltages. The outward current relaxed completely to zero only after hyperpolarizing to potentials more negative than −60 mV. On return to −30 mV from potentials negative to −60 mV, a transient outward current could be seen, which is similar to the A-current reported in these cells (Lledo et al., 1990; Herrington and Lingle, 1994; Sankaranarayanan and Simasko, 1994).

When the cells were exposed to TRH, the outward current at −30 mV was inhibited (Fig. 2, *TRH*). It can be seen clearly, however, that TRH does not influence the transient A-current, which is activated on return to −30 mV from potentials negative to −60 mV. We have found that the cells usually do not recover from the effects of TRH in the time course of our experiments, a result similar to that observed in earlier studies in GH_3 cells (Bauer et al., 1990).

The last set of traces in Figure 2 shows what happened when the cells were exposed to quinpirole after TRH. It can be seen that the outward current at −30 mV is larger after quinpirole. The behavior of this quinpirole-induced current, however, was different from

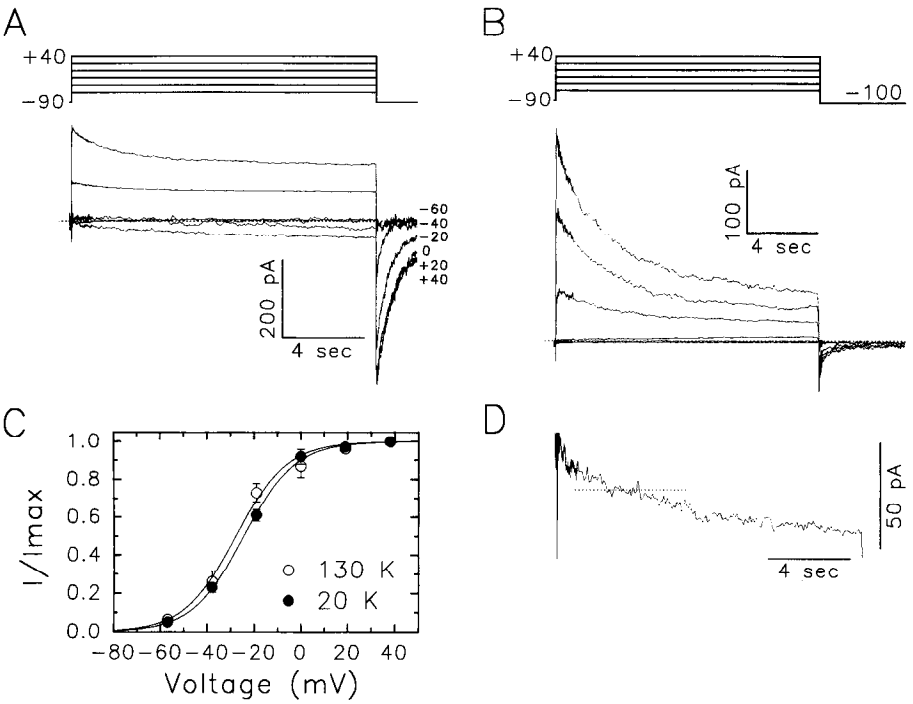

Figure 4. Current–voltage relationship of sustained current component. The sustained currents at 130 K (*A*) and 20 K Ca-free (*B*) bath conditions. The *top traces* illustrate the voltage protocol. The inward currents at the end of depolarizing pulses represent the deactivating tail currents of the sustained-current component. The *numbers* on the *right-hand side* of the traces represent the depolarizing command potential of the respective tail-current trace (*A*). The *dotted lines* in *A* and *B* represent the zero current. *C*, The plot illustrates summarized data of the normalized peak tail current in 130 K (*open circles*, n = 6–11) and 20 K (*filled circles*, n = 6) plotted versus the corresponding command potential. The *lines* are fits to a Boltzmann equation (130 K: V_0 = −28 mV, slope = 11 mV; 20 K: V_0 = −24 mV, slope = 11 mV). *D*, An expanded view of the current in response to the −20 mV command potential from *A*. The *dotted line* indicates 50% of the maximal current.

the current inhibited by TRH in that the current showed no voltage-dependent behavior (compare *Control* with *Quin*, Fig. 2). These results suggest that dopamine and TRH affect different K^+ conductances.

From this experiment it was apparent that under standard physiological conditions TRH inhibited a sustained outward current, which was small in amplitude (~25 pA). To characterize its properties, we needed to alter the recording conditions to amplify this current.

Isolation of resting K^+ current from voltage-dependent and calcium-dependent currents

When depolarized from a holding potential of −80 mV to a potential of +20 mV for 1 sec, lactotrophs under standard bath conditions exhibit a fast activating and rapidly inactivating outward current followed by a slowly growing outward current (Fig. 3*A*). When the bath is changed to Ca^{2+}-free conditions, the slowly growing outward component is lost (Fig. 3*A*). This would indicate that the slowly growing outward current under standard bath conditions is attributable to Ca^{2+}-dependent K^+ currents. What remains in the Ca^{2+}-free bath is a fast activating and rapidly inactivating outward current followed by a sustained component of outward current. To obtain accurate fits to the inactivating portion of outward currents, we increased the duration of depolarization in Ca^{2+}-free bath to 8 sec (Fig. 3*B*). The inactivating current was best fit by the sum of two exponentials plus a sustained offset. The fit is consistent with the fits to voltage-activated currents reported in GH_3 cells (Simasko, 1991a). The inactivating current had time constants of ~80 msec and ~2.5 sec. The fast inactivating component was sensitive to 4-AP, and the slowly inactivating component was sensitive to TEA (data not shown). These inactivating current components have been characterized previously as an A-current (fast inactivating) and a delayed rectifier (slow inactivating) (Lledo et al., 1990; Simasko, 1991a; Herrington and Lingle, 1994; Sankaranarayanan and Simasko, 1994).

In the GH_3 cells, the sustained offset current has been attributed previously to the voltage-dependent properties of the large conductance calcium-activated K^+ channel (Simasko, 1991a) and was found to be insensitive to TRH when activated by strong depolarizing voltages (Simasko, 1991b). Additionally, the current reported to be inhibited by TRH in GH_3 cells has been labeled an inwardly rectifying K^+ current (Bauer et al., 1990) and thus would not be expected to carry outward current. These previous observations suggest that the sustained outward current is not the TRH-sensitive component. On the other hand, without further characterization of its kinetic properties, it is not known at what potential this sustained current will activate. We speculated that this outward current may activate at a potential negative enough that it would be present at the resting membrane potential of these cells and therefore may be the current inhibited by TRH that we observed in Figure 2.

To amplify this component at potentials near the resting membrane potential of these cells, we changed to a high K^+ bath (described in Materials and Methods). Furthermore, we removed Ca^{2+} from the bath (replaced with Mg^{2+}) to avoid calcium currents and activating Ca^{2+}-dependent currents. Finally we delivered 15 sec depolarizing pulses to activate this sustained current and to ensure complete inactivation of the A-current and delayed-rectifier K^+ currents. The characteristics of the sustained current were then investigated by examination of the tail current that followed the return to hyperpolarized potentials after 15 sec depolarizations.

Current–voltage relationship of the sustained current component, revealed in Hi K^+ Ca^{2+}-free conditions

Lactotrophs were held at −90 mV, depolarized to a series of potentials from −60 to +40 mV in 20 mV increments for a duration of 15 sec, and then returned to −90 mV in the Hi K Ca-free bath (Fig. 4*A*). The traces recorded at command potentials negative to 0 mV (−40, −20) had a fast transient inward current that inactivated within milliseconds (difficult to see in Fig. 4 because of the time scale) followed by a slowly growing inward-current component. When the cells are depolarized positive to 0

mV, there is a rapidly activating and inactivating outward current, followed by a sustained current component. At the end of the 15 sec pulse, when the cells are returned to −90 mV, there is a large instantaneous inward current, which is attributable to the sudden increase in driving force on K^+. The tail current at −90 mV represents the deactivation of the sustained-current component. The *open circles* in the current–voltage plot in Figure 4*C* represent the relationship between the normalized peak tail current measured after the cell has been returned to −90 mV (normalized to the peak tail current after the +40 mV depolarization) versus the voltage to which the cells were depolarized. The current–voltage plot shows that this current is maximally activated at 0 mV, because there is no further increase in the tail current when depolarized to more positive potentials. The outward current at the strong depolarizations, however, increases in an approximately ohmic nature. It can also be seen that a substantial portion of this current is present under the normal resting membrane potentials of these cells (−45 to −35 mV). To determine whether the activation voltage was affected by the Hi K bath, we also examined the activation voltage in a 20 mM K^+ bath (Fig. 4*B* and *filled circles* in Fig. 4*C*). In these experiments, however, the cells were returned to a potential of −100 mV after the depolarization to increase the amplitudes of the tail currents. As can be seen, the current activated with similar characteristics in both the 130 mM and the 20 mM K^+ solutions. Because this current is sustained in nature, is half-maximally activated at ~−30 mV, and carries current in both outward and inward directions, it resembles the M-current originally identified in sympathetic ganglionic cells (Brown and Adams, 1980).

We also found the activation of the M-like current to be slow (Fig. 4*D*). In 15 cells, the average time to 50% maximal current at −20 mV depolarization was 1.3 ± 0.2 sec.

The sustained-current component is predominantly K^+-selective

The reversal potential of the sustained-current component was measured by using the following voltage-clamp protocol under bath conditions with $[K^+]_o$ of 130, 20, and 5 mM. The cells were held at −90 mV, depolarized to +20 mV for a period of 15 sec, and then returned to a series of potentials from +10 to −100 mV. The current–voltage relationship of the instantaneous tail currents measured at each potential shows clearly that the sustained current was K^+ selective (Fig. 5). The calculated reversal potential was 0 mV, −47 mV, and −82 mV, and the observed reversal potentials were +4.7 ± 2.4 mV (n = 7), −39.0 ± 2.9 mV (n = 3), and −74.1 ± 0.3 (n = 4) for the solutions with $[K^+]_o$ of 130, 20, and 5 mM, respectively.

Voltage dependence of deactivation of the tail currents

Lactotrophs were held at −90 mV, pulsed to +20 mV for a duration of 15 sec, and then returned to potentials ranging from −60 mV to −100 mV for a period of 9 sec (Fig. 6*A*). Exponential fits to the tail current revealed a fast and a slow component of deactivation (Fig. 6*B*). The tail-current component did not deactivate completely at a potential of −60 mV. At potentials between −80 and −100 mV, the tail currents showed a fast and a slow component of deactivation (Table 1). The time constants for deactivation decreased at more hyperpolarized potentials. Furthermore, in all 11 cells studied, the ratio of the amplitude of the fast to the slow component of tail current increased between deactivation voltages of −80 and −100 mV.

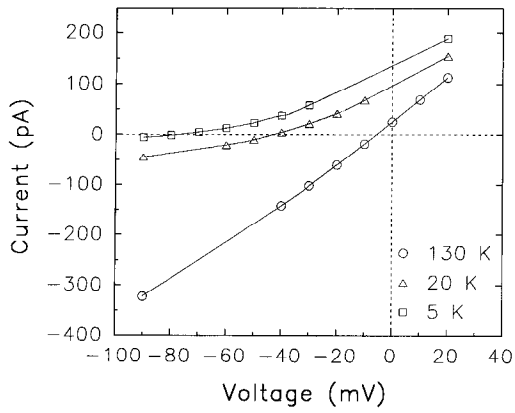

Figure 5. The reversal potential of the instantaneous tail currents at bath K^+ concentrations of 130 mM (*open circles*), 20 mM (*open triangles*), and 5 mM (*open squares*) are illustrated. Data were obtained as described in Results.

Behavior of the two tail components with varying command duration

Exponential fits to deactivating tail currents revealed a fast and a slow component, suggesting the possible existence of two different channels underlying the sustained current. This possibility was tested by examination of the amplitude of the two components after varying the duration of the command potential used to activate the currents. If the two components reflect interconversion between two states of the same channel, then the amplitudes of the components should change reciprocally. On the other hand, if the two components of deactivation are attributable to two distinct channels, then their amplitudes should vary independently of each other.

Lactotrophs were held at −90 mV, pulsed to a potential of +20 mV for durations of 1, 7, 10, 15, and 25 sec in Hi K Ca-free bath, and then returned to the holding potential of −90 mV for a period of 15 sec. Results from a representative cell are shown (Fig. 7*A–C*). It can be seen that at 1 sec the tail-current amplitude is larger than it was at other times and that this increased amplitude is associated with a fast component of deactivation in the tail

Table 1. Results of double-exponential fits to deactivating tail currents

	Amp 1 (pA)	Tau 1 (msec)	Amp 2 (pA)	Tau 2 (sec)	Amp1/Amp2
−80 mV	−223 ± −20	603 ± 73	−216 ± −28	5.4 ± 0.7	1.2 ± 0.2
−90 mV	−339 ± −30	400 ± 47	−171 ± −27	3.2 ± 0.3	2.4 ± 0.5
−100 mV	−432 ± −34	256 ± 29	−151 ± −23	2.9 ± 0.4	3.3 ± 0.5[a]

Summary of the voltage dependence of deactivation kinetics of the tail currents after 15 sec depolarization to +20 mV. The first column is the voltages during the tail current. Amp 1 represents the amplitude of the fast component of deactivation; Tau 1 represents the fast time constant for deactivation; Amp 2 represents the amplitude of the slow component of deactivation; Tau 2 represents the slow time constant for deactivation; and Amp1/Amp2 represents the ratio of the fast to the slow component of deactivation.

[a] Significantly different from −80 mV pulse $p < 0.001$ (paired t test) (n = 11).

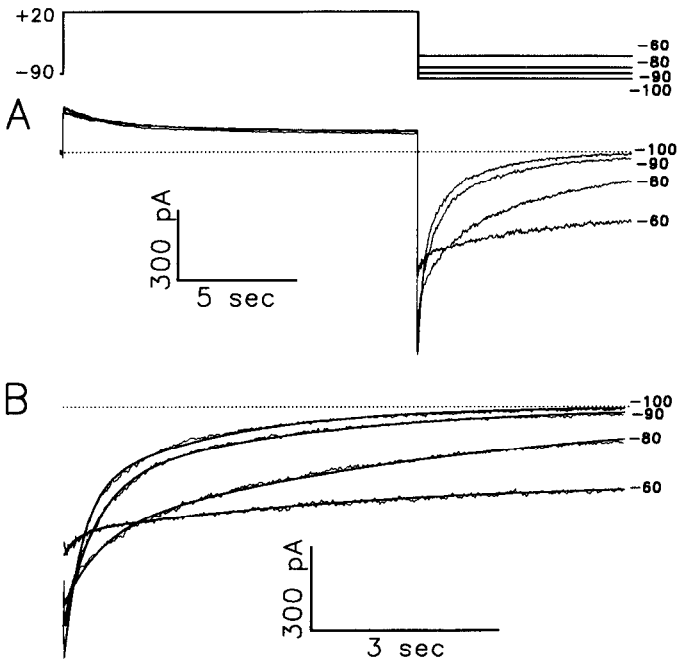

Figure 6. Voltage dependence of tail-current deactivation. *A*, The *top trace* illustrates the voltage protocol. The *middle traces* illustrate the current responses to the voltage protocol. The *dotted line* represents zero current. *B*, Exponential fits to the deactivation of tail currents are illustrated. The *thin wavy lines* represent actual current traces. The *dark line* superimposed on the traces represents the exponential fits to the tail-current deactivation. Respective amplitudes (A) and time constants (τ) are (-60 mV: $A_1 = -77$ pA, $\tau_1 = 204$ msec, $A_2 = -183$ pA, $\tau_2 = 4.2$ sec, offset $= -267$ pA; -80 mV: $A_1 = -264$ pA, $\tau_1 = 596$ msec, $A_2 = -449$ pA, $\tau_2 = 6.6$ sec; -90 mV: $A_1 = -498$ pA, $\tau_1 = 488$ msec, $A_2 = -286$ pA, $\tau_2 = 3.4$ sec; -100 mV: $A_1 = -620$ pA, $\tau_1 = 268$ msec, $A_2 = -322$ pA, $\tau_2 = 2.2$ sec). The *dotted line* represents the zero current.

current. After 7–25 sec of depolarization, the total amplitude of the tail current remains constant. The shape of the tail current, however, changes with increasing time (Fig. 7*B*). Tail currents from pulses of 7–25 sec in duration were best fit by the sum of two exponentials. Fits to the deactivation of the tails after 10 and 25 sec pulses are shown in Figure 7*C*. The time constants for deactivation of the two components did not change appreciably with increasing time, but the relative proportion of the total current accounted for by the slow tail increased with increasing time. This is shown graphically in Figure 7*D*. As can be seen, the total current (sum of the fast and slow components, *filled boxes*) was relatively constant from 7 to 25 sec, but the proportion of the slow-current component of the tail increased from ~30% at 7 sec to >50% at 25 sec. Although the error bars are large because of cell-to-cell variation in the relative amplitude of the slow component, the trend of increasing proportion of the slow component was seen in five of five cells.

The very fast component of tail current after the 1 sec pulse is probably attributable to the deactivation of the delayed rectifier, because A-current would be completely inactivated at this time (τ ~80 msec). To estimate the relative proportion of the slowly deactivating component of M-like current (A_2) to total M-like current ($A_1 + A_2$) at 1 sec, we performed a triple exponential fit on the tail current after the 1 sec pulse (Fig. 7*C*). The very fast component in the tail current after the 1 sec pulse had a deactivation time constant of 10 msec. From this fit, the proportion of the slowly deactivating component of M-like current (A_2) was found to be small compared with the total M-like current ($A_1 + A_2$) (Fig. 7*D*, *open circle* at 1 sec). We found that the sum of A_1 and A_2 (total M-like current) after the 1 sec pulse (to +20 mV command potential) was ~63% of the sum of A_1 and A_2 at 25 sec. This is in close agreement with the previous estimate of the time to 50% activation (1.3 sec at −20 mV; Fig. 4*D*) (it is expected that activation would be slower at less depolarized potentials).

Pharmacology of the M-like current

Lactotrophs were held at −90 mV, pulsed to potentials of −20 and +40 mV for 15 sec, and then returned to −90 mV in Hi K Ca-free bath. The responses of the cells to 1 mM 4-AP and 10 mM TEA are illustrated in Figure 8, *A* and *B*, respectively. The traces on the left represent the entire voltage pulse, whereas the traces on the right represent the first 200 msec of the depolarizing portion of the pulse.

As expected, 4-AP blocked most of the inactivating current at +40 mV (the A-current and the delayed rectifier are both sensitive to 4-AP; Simasko, 1991a). At +40 mV, the sustained current was virtually untouched by 4-AP, an effect that is also reflected in the tail current. At −20 mV, however, 4-AP blocks not only a rapidly inactivating current but also a portion of the sustained current. This block of the sustained current is reflected in the decrease in the tail current after holding at −20 mV in the presence of 4-AP. Thus the M-like current in lactotrophs is sensitive to 4-AP but less so than the A-current is, and the block by 4-AP is voltage dependent.

On the other hand, TEA did not affect the fast-inactivating A-current but did eliminate the slow-inactivating delayed rectifier (Fig. 8*B*), as expected (Simasko, 1991a). TEA did block the sustained current at both −20 mV and +40 mV, with the block greater at −20 mV. This result indicates that although 10 mM TEA has some effect on the M-like current, the M-like current is more resistant to TEA than the delayed rectifier is. Unfortunately, because the M-like current was somewhat sensitive to both TEA and 4-AP, these blockers could not be used to isolate this current further from the A-current and the delayed rectifier.

TRH shifts the voltage of activation to more depolarized potentials

Lactotrophs were held at a potential of −90 mV, pulsed to a series of depolarizing potentials from −60 mV to +40 mV for 15 sec, and then returned to −90 mV under control conditions and after exposure to TRH (Fig. 9). TRH blocked almost all the tail current after −40 mV and a large portion of the tail current after −20 mV command potential; however, TRH produced only a small inhibition of the instantaneous tail current after the more depolarized command potentials. TRH also did not block the transient A-currents and the delayed rectifier-like currents. The plot in Figure 9 shows the relationship of the tail current (normalized to control tail current at +40 mV) versus the command voltage. It can be seen that TRH caused a shift in the activation voltage to more depolarizing potentials (V_0 under control conditions is −28 mV and after TRH is −17 mV).

To characterize the effect of TRH on tail-current deactivation kinetics, tail currents of 15 sec duration were recorded after depolarization to −20 and +20 mV command potentials. Exponential fits to the deactivation of the tail currents under control conditions and after TRH exposure were performed. TRH caused a decrease in the time constants for deactivation of both the fast

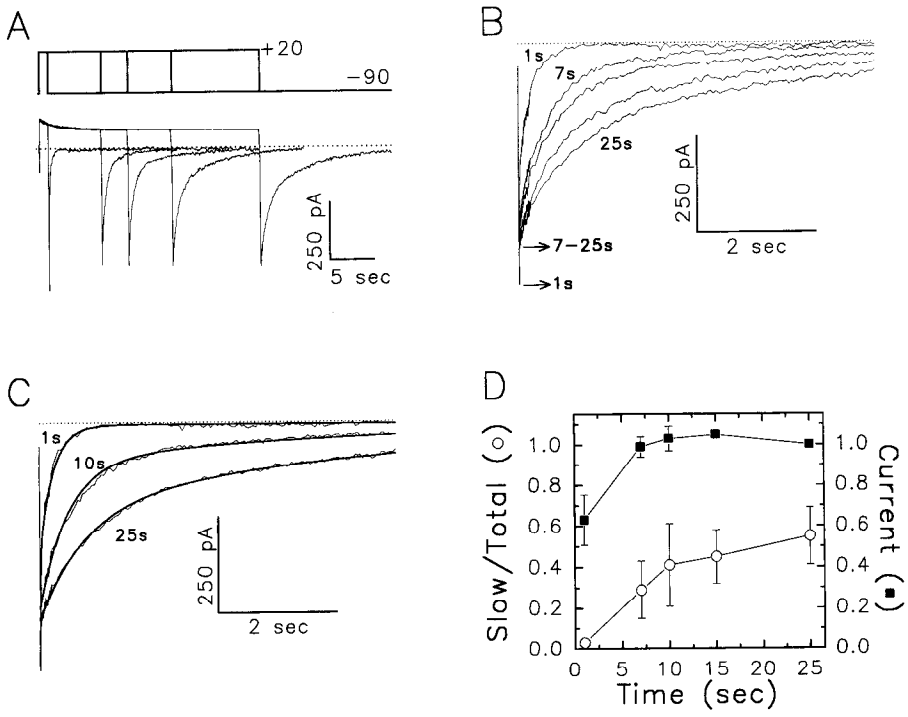

Figure 7. Effects of increasing the duration of depolarization on the two tail-current components. *A*, The *top trace* illustrates the voltage protocol (holding potential −90 mV, followed by a command potential to +20 mV for durations of 1, 7, 10, 15, and 25 sec followed by return to −90 mV). The traces show the outward current on depolarization followed by tail currents on repolarization. The *dotted line* represents zero current. *B*, The traces illustrate 6 sec of the deactivation of the tail-current components after 1, 7, 10, 15, and 25 sec depolarizations. The *traces* labeled *1s*, *7s*, and *25s* are the tail-current components after 1, 7, and 25 sec of depolarization. The *arrows* represent amplitude of the peak tail current after the 1 sec depolarization (*1s*) and that after 7, 10, 15, and 25 sec depolarization (*7–25s*). The *dotted line* represents zero current. *C*, Fits to the tail-current component after 1, 10, and 25 sec depolarization are illustrated. The *thin wavy line* represents the actual traces, whereas the *solid line* represents the exponential fits to the tail-current component (1 sec tail: $A_{\text{very fast}} = -389$ pA, $\tau_{\text{very fast}} = 10$ msec, $A_1 = -340$ pA, $\tau_1 = 231$ msec, $A_2 = -5$ pA, $\tau_2 > 20$ sec; 10 sec tail: $A_1 = -384$ pA, $\tau_1 = 487$ msec, $A_2 = -101$ pA, $\tau_2 = 5.6$ sec; 25 sec tail: $A_1 = -293$ pA, $\tau_1 = 783$ msec, $A_2 = -222$ pA, $\tau_2 = 6.2$ sec) The labels *1s*, *10s*, and *25s* represent the tail currents after 1, 10, and 25 sec of depolarization. The *dotted line* represents the zero current. *D*, The relation between the amplitude of the slow tail-current component (A_2) to amplitude of the total-tail current attributable to the M-like current component (sum of $A_1 + A_2$) (*left vertical axis, open circles*) is plotted versus duration of depolarization (*Time*). The *right vertical axis* represents the total M-like tail-current amplitude (*filled squares*) at each time normalized to the total M-like tail-current amplitude at 25 sec. The data points and error bars in the figure are the mean ± SE of five cells.

and the slow components of tail current after both −20 and +20 mV command potentials (Table 2). In addition, it can be seen that the block of the M-like current by TRH was voltage dependent, being greater at −20 mV than at +20 mV.

Effect of TRH but not TEA is lost in whole-cell recording situations

Lactotrophs were held at −90 mV, pulsed to a command potential of −20 mV, and then returned to −90 mV for a period of 15 sec under control conditions and after 1 μM TRH in the perforated-patch and whole-cell mode of patch-clamp recording techniques (Fig. 10, *A1* and *A2*, respectively). Illustrated in Figure 10 are 5 sec of the tail currents after the command pulse. TRH inhibited the tail current and in addition altered the deactivation kinetics of the tail in the perforated-patch recording situation (Fig. 10*A1*). In three of five cells examined in the whole-cell recording mode, however, TRH had no effect (Fig. 10*A2*). In the remaining two cells, the effect of TRH to inhibit the M-like current was reduced significantly (data not shown). Furthermore, when 0.5 mM GDP-β-S was added to the standard whole-cell pipette solution, TRH had no effect on the tail current in three of three cells (data not shown).

In contrast to the failure of TRH to act in the standard whole-cell mode, TEA inhibited the tail current in both the perforated-patch recording situation and the standard whole-cell configuration (Fig. 10, *B1* and *B2*, respectively). This suggests that unlike TRH, which probably induces a G-protein-coupled or second

Table 2. Effect of TRH on double-exponential fits to deactivating M-like currents

	−20 mV (n = 4)		+20 mV (n = 6)	
	Control	TRH	Control	TRH
Amp 1 (pA)	−379 ± −49	−277 ± −52[a]	−392 ± −33	−346 ± −36
Tau 1 (msec)	500 ± 89	179 ± 18[b]	554 ± 42	216 ± 18[b]
Amp 2 (pA)	−122 ± −37	−107 ± −25[a]	−168 ± −50	−165 ± −35[a]
Tau 2 (sec)	4.2 ± 0.4	2.4 ± 0.3[a]	7.6 ± 3.6	3.4 ± 0.7[a]

Summary of the effect of TRH on deactivation of tail-current component after −20 mV and +20 mV depolarization. The columns −20 mV and +20 mV show the tail-current characteristics under control conditions and after TRH, respectively. Refer to Table 1 for abbreviations.

[a] Significantly different from control (paired *t* test), $p < 0.05$.

[b] Significantly different from control (paired *t* test), $p < 0.01$.

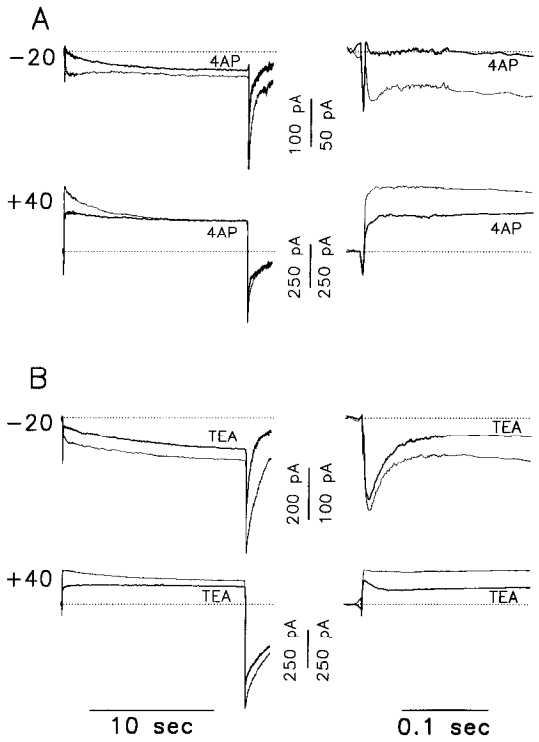

Figure 8. Pharmacology of the M-like current. Cells were held at −90 mV, depolarized to −20 and +40 mV for a duration of 15 sec, and then returned to −90 mV. The *left traces* represent the entire duration of the voltage pulse. The *right traces* illustrate the first 200 msec of the depolarizing pulse of the corresponding *left trace*. *A*, The effect of 1 mM 4-AP (*4AP*) on the currents elicited at the indicated potentials. *B*, The effect of 10 mM *TEA*. For each set of traces, the *dark traces* represent the trace after exposure to TEA or 4-AP, whereas the *lighter trace* represents the control trace. The *scale bar* for each of the sets of figures is indicated in the *middle*. The *dotted line* represents zero current in all sets of traces.

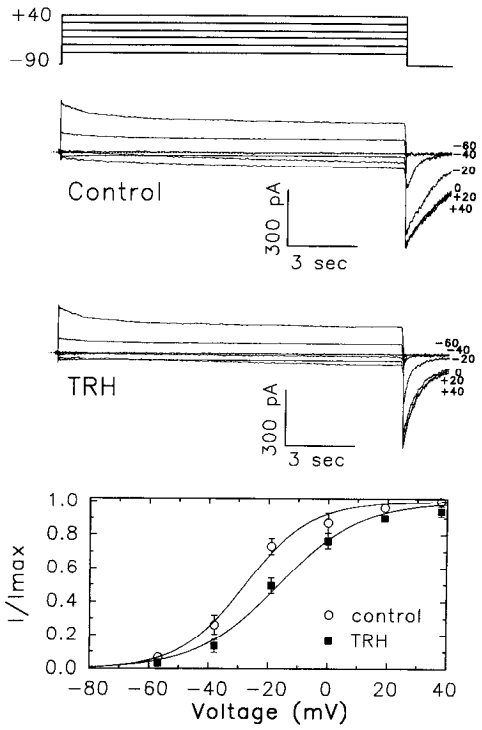

Figure 9. Effects of TRH on the voltage of activation. The *top trace* illustrates the voltage protocol (the holding potential is −90 mV) followed by a series of depolarizations from −60 to +40 mV and then returned to a potential of −90 mV. The traces marked *Control* and *TRH* are the traces before and after TRH exposure, respectively. The *numbers on the right* of the two sets of traces indicate respective depolarizing pulses in control conditions and after TRH exposure. The *dotted line* in the traces represents zero current. The *plot in the bottom* represents the relation between the peak tail current normalized to the peak tail current after the control +40 command potential (*I/Imax*) versus the corresponding command potential. The *open circles* represent the control data points, and the *filled squares* represent the data points after TRH exposure. The *error bars* represent the SE from a mean of 5–11 cells. The *lines on the graph* are from fits to a Boltzmann equation (control: V_0 = −28 mV, slope = 11 mV; TRH: V_0 = −17 mV, slope = 14 mV).

messenger-mediated effect on the M-like current, TEA probably produces an effect directly on the channel.

M-like current in the GH_3 cells

The above observations that TRH modulates an M-like current in primary rat pituitary lactotrophs led us to question whether the TRH-modulated current previously characterized as an inwardly rectifying current in the clonal GH_3 cells was actually an M-like current (Bauer et al., 1990). To answer this question, voltage-clamp experiments were performed on GH_3 cells in a Hi K Ca-free bath (Fig. 11). Cells were held at −70 mV, pulsed to various command potentials for a duration of 15 sec, and then returned to a potential of −70 mV under control situations (Fig. 11*A*) and after exposure to 10 mM TEA (Fig. 11*D*) and 1 mM 4-AP (Fig. 11*E*).

In response to a depolarization to +40 mV, the current showed a fast transient outward-current (A-current) component, a slowly inactivating outward-current (delayed-rectifier current) component, and a sustained outward-current component, which have been described earlier (Simasko, 1991a). At the end of the depolarizing pulse, when the cells were returned to −70 mV, there was a tail current that was very small when the depolarizing pulse was −40 mV but reached maximal activation at 0 mV (Fig. 11*C*). The activation voltages for these tail currents from three cells are summarized in Figure 11*B*. The 50% activation at −24 mV agrees quite well with that observed in lactotrophs (Fig. 4*C*). Similar to that in lactotrophs, this current was outward at potentials positive to the K^+ reversal potential.

Also similar to the M-like current found in lactotrophs, the M-like current in GH_3 cells had a biphasic deactivation (Fig. 11*C*), and the current was resistant to 10 mM TEA (Fig. 11*D*) and 1 mM 4-AP (Fig. 11*E*). Evidence for an M-like current (maximal activation at 0 mV, sustained outward currents at potentials positive to E_K, and biphasic deactivations) was found in 12 of 12 cells examined. The relative resistance of the M-like currents to TEA and 4-AP was observed in seven of seven and four of four cells tested, respectively. These observations demonstrate that the conducting properties, kinetic behavior, and pharmacological sensitivity of the sustained current in GH_3 cells are similar to those of the M-like current in lactotrophs.

DISCUSSION

In this study we have characterized a sustained K^+ current in rat lactotrophs that is modulated by TRH. This current has characteristics similar to the M-current originally described by Brown and Adams (1980). The M-like current described here is different from the voltage-dependent K^+ currents in rat lactotrophs described previously (Lledo et al., 1990; Herrington and Lingle, 1994). The kinetic and pharmacological properties of this current

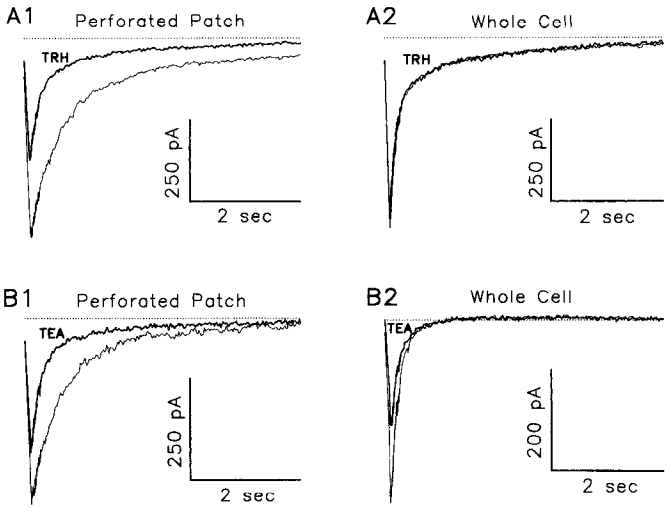

Figure 10. Effect of TRH but not TEA is lost in whole-cell recording conditions. The traces illustrate 5 sec of tail currents at −90 mV after 15 sec depolarization to −20 mV from a holding potential of −90 mV. The *lighter traces* represent the control conditions, whereas the *darker traces* are those after exposure to 1 μM *TRH* (*A1, A2*) or 10 mM *TEA* (*B1, B2*), respectively. The *dotted line* represents zero current in all sets of traces.

have been characterized, and the effect of TRH on this current has been described. Our results suggest that the ability of TRH to shift the activation voltage of this current in a depolarizing direction underlies the increased excitability caused by TRH in lactotrophs.

Identity of lactotrophs used in this study

Cells used in this study were from pituitary cultures enriched with lactotrophs. Consistent with previous studies (Mason and Ingram, 1986; Burris and Freeman, 1993), we found that ~70% of the cells in our cultures contained prolactin, as determined by immunohistochemistry. An additional confirmation of the identity of these cells as lactotrophs is that 90% of the cells challenged with quinpirole hyperpolarized (Chen et al., 1989). The failure of TRH to act on 5 of 20 cells in current-clamp experiments could be attributable to the possibility that the five nonresponding cells were not lactotrophs, although we have no evidence that this is so. Interestingly, almost every cell examined had the M-like current we have characterized in this study. Thus, expression of the M-like current may not be a lactotroph-specific property. On the other hand, on the basis of our study we can state with confidence that most, if not all, rat lactotrophs express an M-like current.

TRH on membrane-potential behavior

The depolarizing effect of TRH on membrane-potential behavior in current-clamp studies correlates well with results published earlier (Ingram et al., 1986; Sartor et al., 1990). We found, however, that TRH did not consistently produce the initial rapid hyperpolarization that has been reported in GH_3 cells. One possibility is that the calcium release from intracellular stores could be smaller in lactotrophs than in the GH_3 cells. Alternatively, we have consistently found smaller calcium-activated K^+ currents in normal lactotrophs than in the GH_3 clonal cells (unpublished observation). Thus, in many cells the depolarizing influence of TRH may mask a small initial hyperpolarization.

TRH modulates an M-like current

It has been suggested that the TRH-induced increase in excitability in GH_3 cells is produced by inhibition of voltage-dependent K^+

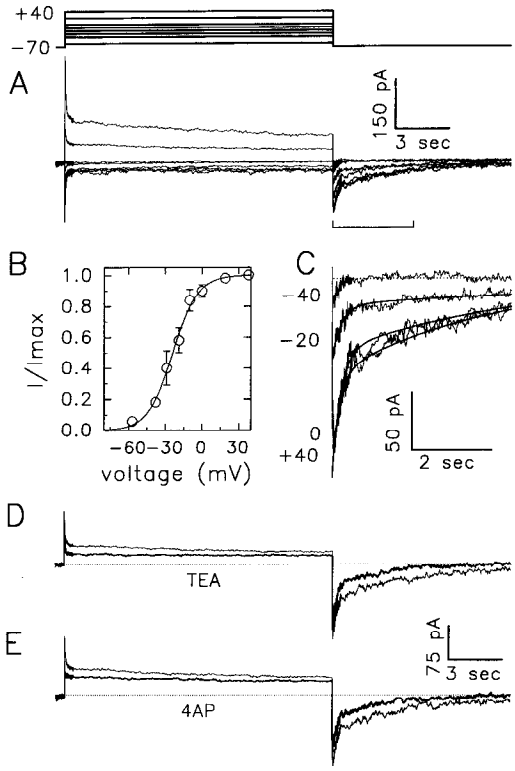

Figure 11. M-like current in GH_3 cells. The *top traces* illustrate the voltage protocol. *A*, Current traces in 130 K Ca-free bath. *B*, The normalized peak tail current (normalized to the tail current after the +40 mV command potential) versus the corresponding command potential ($n = 3$). The *line* is a fit to a Boltzmann equation ($V_0 = -24$ mV, slope = 10 mV). *C*, The *thin wavy lines* represent the tail currents, and the *dark lines* represent double-exponential fits to tail currents after command potentials of −20, 0, and +40 mV (−20 mV: $A_1 = -46$ pA, $\tau_1 = 238$ msec, $A_2 = -23$ pA, $\tau_2 = 9.1$ sec; 0 mV: $A_1 = -108$ pA, $\tau_1 = 208$ msec, $A_2 = -65$ pA, $\tau_2 = 4.8$ sec; +40 mV: $A_1 = -84$ pA, $\tau_1 = 189$ msec, $A_2 = -85$ pA, $\tau_2 = 4.0$ sec). *D*, Effect of 10 mM *TEA* at +20 mV command potential. The *dark trace* represents the trace after exposure to TEA. *E*, Effect of 1 mM 4-AP (*4AP*) at +20 mV command potential. The *dark trace* represents the trace after exposure to 4-AP. The *dotted lines* in *A* and *C–E* represent the zero current.

currents (Dubinsky and Oxford, 1985) or of an inwardly rectifying K^+ current (Bauer et al., 1990; Barros et al., 1992). The results of our present studies agree with earlier observations that TRH does not modulate an A-current or a delayed rectifier-like current (Bauer et al., 1990; Simasko, 1991b). Our voltage-clamp experiments, however, clearly show that the current inhibited by TRH is an outward current and not an inward rectifier, as has been suggested in GH_3 cells (Bauer et al., 1990; Barros et al., 1992). Characterization of the current modulated by TRH shows that it has no inactivation, begins to activate at ~−60 mV, and is almost completely activated at ~0 mV, all of which are characteristics of M-current (Brown, 1988a). Shifting the activation of this current in a depolarizing direction would remove a hyperpolarizing influence at the interspike voltages in lactotrophs (−45 to −35 mV) and lead to increased excitability. This increased excitability would lead to more Ca^{2+} influx and contribute to the sustained release of prolactin caused by TRH (Gershengorn, 1986).

This conclusion led us to question how this M-like current we have characterized in lactotrophs compares with the inwardly rectifying K^+ current thought to be the target of TRH action in GH_3 cells (Bauer et al., 1990; Barros et al., 1992). We believe that

the two currents are identical but that the current in GH_3 cells was misidentified as inwardly rectifying, because it was never really studied at voltages that would produce outward currents. Our study of GH_3 cells confirms our view that the current in GH_3 cells and in lactotrophs is an M-like current. Previous investigators found that TRH-regulated current is completely blocked by 10 mM 4-AP and 10 mM TEA (Bauer et al., 1990), whereas we found the TRH-regulated current to be somewhat resistant to these blockers. Possible explanations for these disagreements may be the lower concentration of 4-AP in our study (1 mM vs 10 mM) or the membrane voltage at which these potential-sensitive agents were examined (much more hyperpolarized in previous reports).

A final issue is reconciliation of previous observations that TRH inhibits voltage-dependent K^+ currents in GH_3 cells (Dubinsky and Oxford, 1985) versus other observations that claim it does not (Bauer et al., 1990; Simasko, 1991b). The effects of TRH in the study by Dubinsky and Oxford (1985) were examined at a test potential of +20 mV, and the reduction in outward current is mostly of a sustained nature. Because we observed a small effect of TRH on currents measured at +20 mV, it could be that the current inhibited in the study by Dubinsky and Oxford (1985) is in fact the M-like current. On the other hand, in the study by Simasko (1991b) the effects of TRH were examined at very depolarized potentials (+60 or more). Because we have observed that TRH mainly shifts the activation voltage of the M-like current with only a small inhibition of the current amplitude at more depolarized potentials, it is not surprising that TRH had virtually no effect at very depolarized potentials.

Comparison of M-like currents in lactotrophs versus other cell systems

M-current was originally identified in frog sympathetic neurons as the current inhibited by muscarinic agonists, thus causing increased excitability (Brown and Adams, 1980). It has subsequently been described in numerous neuronal preparations and stomach smooth muscle (for review, see Brown, 1988b). We are the first to report an M-like current in pituitary cells. The characteristics of this current are similar to those reported earlier (no inactivation, half-maximal activation V_0 of −28 mV, and a slope factor of 11 mV vs V_0 of −35 mV and a slope factor of 10 mV reported in frog sympathetic ganglia; Brown, 1988a). Exponential fits to the deactivation of M-current in lactotrophs revealed a fast and a slow component of deactivation similar to those of the intermediate and slow components of deactivation reported earlier in frog sympathetic ganglia (Marrion et al., 1992), except that the time constants of deactivation in lactotrophs are slower by a factor of 4–5. In lactotrophs, both rates of deactivation increased with greater hyperpolarization in contrast to only the intermediate rate increasing in frog sympathetic ganglia. As with M-current in frog sympathetic ganglia, we also found the ratio of the amplitude of the fast to slow component of deactivation to increase with more hyperpolarizing potentials (Marrion et al., 1992). Finally, M-like currents in lactotrophs were incompletely blocked by 1 mM 4-AP and 10 mM TEA, similar to results in bullfrog sympathetic ganglia (Adams et al., 1982; Brown, 1988a).

An additional property of the M-like current in pituitary cells not reported in neuronal preparations is that the current accumulates in the slowly deactivating state with increasing duration of depolarizations. Our results are consistent with a model of two interconverting open states of the channel that underlies M-like current, but how such a model would explain other aspects of the current (e.g., increasing ratio of fast to slow deactivation with more hyperpolarizing potentials) needs to be investigated.

We also found that the manner in which TRH modulates the M-like current is different from muscarinic modulation in neurons. Although TRH caused a shift in the voltage of activation to more depolarized potentials and increased the rates of deactivation of both the fast and the slow components, muscarinic modulation of the current only decreases the amplitude and does not influence the rates of deactivation (Marrion et al., 1992). In gastric smooth muscle cells, however, isoproterenol modulates M-currents by not only causing a hyperpolarizing shift in the activation voltage but also by decreasing the rate of deactivation of M-currents (Sims et al., 1990). Thus, TRH modulates the pituitary M-like current in a manner exactly opposite to that of isoproterenol in gastric smooth muscle.

Comparison with TRH-modulated K^+ currents in other systems

TRH is known to influence the excitability of spinal motoneurons (Nicoll, 1977; White, 1985). It has been suggested that the mechanism of increase in excitability by TRH is attributable to modulation of Ba^{2+}-sensitive and Ba^{2+}-insensitive potassium conductances in hypoglossal motoneurons (Rekling, 1990; Bayliss et al., 1992) and lumbar motoneurons (Fisher and Nistri, 1993). These studies have also suggested that TRH increases a cationic conductance. In a perforated patch-clamp study performed on dissociated rat hippocampal CA1 neurons, there is evidence supporting an action of TRH to inhibit an M-current (Ebihara and Akaike, 1993). Our observation that TRH modulates an M-like current correlates well with this latter study.

Conclusions

We have identified and characterized the kinetic properties of an M-like current in rat lactotrophs that is modulated by TRH. This is the first report of an M-like current in pituitary cells. The properties of this current suggest that it is one of the predominant conductances active under the resting membrane conditions of rat lactotrophs. The size of this current under standard physiological conditions is small, but because the input resistance of these cells is high (5–10 GΩ), inhibition of even a small current will lead to a pronounced change in the membrane potential.

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