Isolation of a Long-Lasting *eag*-Related Gene-Type K^+ Current in MMQ Lactotrophs and Its Accommodating Role during Slow Firing and Prolactin Release

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Native rat lactotrophs express thyrotrophin-releasing hormone-dependent K^+ currents consisting of fast and slow deactivating components that are both sensitive to the class III antiarrhythmic drugs that block the *eag*-related gene (ERG) K^+ current (I_{ERG}). Here we describe in MMQ prolactin-releasing pituitary cells the isolation of the slowly deactivating long-lasting component (I_{ERGS}), which, unlike the fast component (I_{ERGF}), is insensitive to verapamil $2 \mu M$ but sensitive to a novel scorpion toxin (ErgTx-2) that hardly affects I_{ERGF} . The time constants of I_{ERGS} activation, deactivation, and recovery from inactivation are more than one order of magnitude greater than in I_{ERGF} , and the voltage-dependent inactivation is left-shifted

by ~ 25 mV. The very slow MMQ firing frequency (~ 0.2 Hz) investigated in perforated patch is increased approximately four times by antiarrhythmic agents, by ErgTx-2, and by the abrupt I_{ERGS} deactivation. Prolactin secretion in the presence of antiarrhythmics is three- to fourfold higher in comparison with controls. We provide evidence from I_{ERGS} and I_{ERGF} simulations in a firing model cell to indicate that only I_{ERGS} has an accommodating role during the experimentally observed very slow firing. Thus, we suggest that I_{ERGS} potentially modulates both firing and prolactin release in lactotroph cells.

Key words: K^+ channels; lactotrophs; firing; anterior pituitary cells; erg genes; prolactin release

Thyrotrophin-releasing hormone (TRH) induces a biphasic increase in prolactin secretion with a brief initial hyperpolarization (attributable to Ca^{2+} -dependent K^+ channels) and a long-lasting depolarization (Ozawa and Sand, 1986). Experiments in GH₃ or GH₃/B₆ tumor-derived pituitary cells have revealed that a TRH-dependent inward-rectifying current (Bauer et al., 1990; Barros et al., 1992, 1994) is sustained by *eag*-related gene (ERG) channels (Barros et al., 1997; Bauer, 1998). Studies of TRH action and prolactin release have also been performed in native pituitary cells (Corrette et al., 1996; Sankaranarayanan and Simasko, 1996; Schäfer et al., 1999), but the current in these cells is characterized by a double exponential deactivation with time constants of the order of 5–6 sec, a left-shifted voltage-dependent activation with

respect to ERG K^+ current (I_{ERG}), and a slightly different sensitivity to the drug E-4031.

ERG potassium channels (Warmke and Ganetzky, 1994; Trudeau et al., 1995; Titus et al., 1997) regulate the duration of heart action potential (Sanguinetti et al., 1995) and also sustain spike-frequency adaptation in neurons (Chiesa et al., 1997) and human pancreatic β -cells (Rosati et al., 2000). The peculiar properties of the ERG activation gate (Schönherr et al., 1999) are such that they lead to the accumulation of an outward I_{ERG} that is sufficient to inhibit firing.

The experiments described here were performed using the pituitary tumor-derived MMQ cell line, which, like native lactotrophs, secretes prolactin and is responsive to the inhibitory action of dopamine (Judd et al., 1988). We show that the properties of the MMQ inwardly rectifying current are very similar to those found in normal lactotrophs (Sankaranarayanan and Simasko, 1996; Schäfer et al., 1999) insofar as they have a fast-deactivating component (I_{ERGF}) and a long-lasting component (I_{ERGS}), both of which are sensitive to typical ERG inhibitors such as WAY 123,398, E-4031, ErgTx-1 (Gurrola et al., 1999), and caffeine (Barros et al., 1997). The fast and slow deactivating components can be distinguished by a novel scorpion toxin that specifically blocks only ERGS, by verapamil, an ERG blocker that blocks ERGF but does not affect ERGS (up to $2\text{--}3 \mu M$), and by using a biophysical approach; furthermore, the $[K^+]_o$ dependencies of the two components are different. The functional role of ERGF and ERGS components was investigated by current

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clamping MMQ cells in the perforated whole-cell mode and applying various pharmacological and biophysical manipulations. We also determined the crucial role of I_{ERGS} in ultra-slow firing by means of a model reconstruction of its biophysical properties. Finally, we investigated the dependence of prolactin release on ERGF/ERGS blockers and found, by using the RNase protection technique, that all three of the known *erg* genes are expressed in these cells, albeit at different levels.

Some of the preliminary results have been published previously in abstract form (Rosati et al., 1998).

MATERIALS AND METHODS

Cell culture. The MMQ pituitary cell line (kindly provided by Dr. I. S. Login, University of Virginia School of Medicine, Charlottesville, VA) was cultured in RPMI-1640 medium containing 7.5% horse serum (Invitrogen) and 2.5% FCS (Euroclone).

Solutions and drugs. The standard extracellular solution contained (in mM): NaCl 130, KCl 5, CaCl_2 2, MgCl_2 2, HEPES-NaOH 10, D-glucose 5, pH 7.40. In the high K^+ external solution ($[K^+]_o = 40$ mM), NaCl was replaced by an equimolar amount of KCl. The standard pipette solution at $[Ca^{2+}]_i = 10^{-7}$ M (pCa 7) contained (in mM): K^+ -aspartate 130, NaCl 10, MgCl_2 2, CaCl_2 1.3, EGTA-KOH 10, HEPES-KOH 10, ATP (Mg^{2+} salt) 1, pH 7.30.

The pipette solution used for the perforated patch experiments contained (in mM): K^+ -aspartate 140, NaCl 10, MgCl_2 2, HEPES-KOH 10, amphotericin B (Invitrogen) 150 $\mu\text{g}/\text{ml}$, pH 7.30. The action potentials (APs) and firing were initially recorded in current clamp by means of an Axopatch 200A in I_{fast} mode (Axon Instruments, Foster City CA) and subsequently by means of the MultiClamp 700A, which makes a perfect voltage recording instead of adapting a patch amplifier to current clamp (Magistretti et al., 1996). We used WAY-123,398 (Spinelli et al., 1993) (WAY) as a specific ERG blocker, although all of its effects can also be obtained using E-4031 (Faravelli et al., 1996). WAY (from Dr. W. Spinelli, Wyeth-Ayerst Research, Princeton, NJ) was dissolved in distilled water to make 5 mM stock solutions. E-4031 was a generous gift from Sanofi Recherche (Montpellier, France). In addition to E-4031 and WAY-123,398 we also used haloperidol (Sigma, St. Louis, MO), terfenadine, and astemizole (kindly provided by Prof. Maurizio Tagliatella, University of Naples, Naples, Italy).

Purification and chemical analysis of Ergtoxin-2. The novel Ergtoxin-like peptide used in this study was purified from the Mexican scorpion *Centruroides noxius* Hoffmann by means of a combination of chromatographic separations, starting with Sephadex G-50 gel filtration, followed by two steps of HPLC. In the last chromatographic step, the peptide elutes as a single symmetrical component. When submitted to automatic Edman degradation, it gives a unique sequence with the three most N-terminal amino acids: Gly-Arg-Asp. The molecular mass obtained in a Finnegan LCQ-DUO mass spectrometer was 4783. The fact that only one signal was obtained showed that the peptide was homogeneous. This peptide has 43 amino acid residues and contains 4 disulfide bridges (G. Gurrola, L. D. Possani, unpublished observations). It is different from the Ergtoxin described previously by our group (Gurrola et al., 1999) and was called Cn ErgTx-2: Cn stands for *C. noxius*, and the 2 indicates that it is the second such peptide to be fully characterized from this venom.

Patch-clamp recordings and data analysis. The currents were recorded at room temperature as described previously (Faravelli et al., 1996) with 4–6 M Ω pipette resistance; cell capacitance and series resistance errors were carefully compensated for (85–90%) before each voltage-clamp protocol run. The extracellular solutions were delivered through a nine-hole (0.6 mm) remote-controlled linear positioner, with an average response time of 2–3 sec, placed near the cell under study. The ERG and ERGS inactivation curves were obtained by plotting the normalized peak chord conductance ($G_{\text{peak}} = I(\text{peak})/(V_M - E_K)$) against membrane potential V_M . To compensate for the deactivation present at the peak (especially at membrane potentials negative to -80 mV), we extrapolated the exponential decaying fitting of deactivation to the onset of the test pulse (Smith et al., 1996). To study voltage-dependent activation, the peak tail currents elicited at -120 mV were normalized to maximum and plotted against the preconditioning voltage. pClamp 8 (Axon Instruments) and Origin 4.1 (Microcal Inc.) software were used routinely during data acquisition and analysis.

Prolactin release. Prolactin release was assayed using an enzyme immunoassay (EIA) system (Amersham Biosciences, Milano, Italy). Briefly,

the cells were incubated for 1 hr with the test substances, and then the medium was collected and stored at -80°C until assay. The amount of prolactin released by the cells was measured by evaluating the competition between the hormone present in the samples and a fixed quantity of biotin-labeled rat prolactin for a limited amount of rat prolactin antibody immobilized on precoated microtiter wells. The actual concentration of prolactin in the samples was evaluated by comparing the obtained results with those derived from a standard curve prepared using known concentrations of rat prolactin standards.

Molecular biology. The RNase protection assays (RPAs) were performed according to Dixon and McKinnon (1994), with some modifications. Briefly, total RNA was extracted from freshly collected rat brains and retinas or semiconfluent MMQ cell cultures using the guanidinium/isothiocyanate method (Maniatis et al., 1989), and 50 μg was hybridized overnight at 48°C with ^{32}P -UTP-labeled RNA probes. The rat *erg* probes used for these experiments were the same as those used by Shi et al. (1997), linearized with *Hind*III and transcribed with T3 polymerase. Rat *cyclophilin* (Ambion) was used as an internal loading control. Digestion with RNase A (40 $\mu\text{g}/\text{ml}$) and T1 (2 $\mu\text{g}/\text{ml}$) was then performed for 1 hr at room temperature. Five micrograms of yeast tRNA were used as a negative control for the probe self-protection bands. Finally, the samples were run on a 6.6% polyacrylamide gel and exposed for between 1 and 7 d.

Model. The analysis shown in Figures 2C (*inset*) and 8 were obtained using the Axon Engineer Pro program (AEON Software, Madison, WI) and followed the classical scheme (Shibasaki, 1987; Sanguinetti and Jurkiewicz, 1990) for interpreting I_{K^+} . This scheme describes I_{ERGF} and I_{ERGS} as the product of the two voltage-dependent variables, which are here called $a(V)$ (activation) and $h(V)$ (inactivation) according to the equation: $I = g_{\text{max}} a(V) h(V) (V_M - E_K)$, where g_{max} is the maximal conductance of the fully open channels (Faravelli et al., 1996). The I_{ERGS} inward currents predicted by this model are shown in Figure 2C (*inset*, last panel).

To minimize the number of conductances and create the continuous firing of heart-like action potentials, we used the interplay of voltage-dependent I_{Ca} and I_{Ks} currents having properties that are of no great concern here. As explained in Results, to annul the activation variables of ERGF and ERGS, we forced the holding potential at -60 mV at the beginning of the experiment so that the development of the action of either ERGF or ERGS could be easily seen. The maximal conductance ratios were the following: $g_{\text{Ks}}/g_{\text{Ca}}/g_{\text{Leak}}/g_{\text{ERGF}}/g_{\text{ERGS}} = 1.0:17:0.002:0.011:0.044$. The reversal potential for I_{Leak} was set to -55 mV. We used the classical formalism of Hodgkin and Huxley with the following forward (α) and backward (β) rate constants for ERGF and ERGS models: $\alpha_{\text{ERGF}} = 2e-006\exp[0.07(V + 85)]$; $\beta_{\text{ERGF}} = 0.11/\{1 + \exp[0.085(V + 125)]\} + 2e-005$; $\alpha_{\text{hERGF}} = 10/\{1 + \exp[0.045(V + 190)]\}$; $\beta_{\text{hERGF}} = 0.05/\{1 + \exp[-0.05(V + 100)]\}$; $\alpha_{\text{ERGS}} = 1e-005/\{1 + \exp[-0.15(V + 20)]\}$; $\beta_{\text{ERGS}} = 1e-005\exp[-0.08(V + 65)]$; $\alpha_{\text{hERGS}} = 40\exp[-0.048(V + 255)]$; $\beta_{\text{hERGS}} = 0.040/\{1 + \exp[-0.04(V + 50)]\}$.

RESULTS

The inwardly rectifying current of MMQ cells can be separated into a quickly and a slowly deactivating component with a different sensitivity to verapamil

As in native lactotrophs (Schäfer et al., 1999), the inwardly rectifying current of MMQ cells consists of a transient component and a long-lasting component, both of which are inhibited by 1 μM E-4031 and WAY123,398, as well as by other ERG blockers such as haloperidol (2 μM), astemizole (1 μM), and terfenadine (2 μM). In native lactotrophs, the long-lasting component can be deactivated after prolonged hyperpolarization to -120 mV for 10 sec, and it reappears only after a depolarizing pulse to 0 mV for 10 min (Schäfer et al., 1999).

The ERG-like K^+ currents in MMQ cells were recorded using a $[K^+]_o$ of 40 mM (see Materials and Methods). The currents were elicited from a holding potential of 0 mV, at which the channels are open but completely inactivated, to test potentials (V_T) in the -20 to -120 range (Fig. 1, *H*, *inset*). Consistently, they are normally seen as inward tail currents that reflect the rapid removal of inactivation, which is normally finished at peak, and a

Figure 1. Biophysical and pharmacological separation of ERGF and ERGS components from MMQ cell currents.

A, Superimposed recordings elicited with the protocol shown below before (■) and after 30 sec conditioning at -100 mV (●), and after perfusion with $1 \mu\text{M}$ WAY-123398 (way, ▲). Note different symbols to locate traces. **B**, **C**, Superimposed recordings elicited with the protocol shown below before (■), after the application of $3 \mu\text{M}$ verapamil (ver, ●), and their difference verapamil-sensitive (■–●), after washout (▲), and after 30 sec conditioning at -100 mV (▼), and their difference (▲–▼). The unlabeled trace in **B** is obtained by subtracting the verapamil-sensitive trace labeled ■–● multiplied by 1.6 from trace ●. **D**, Verapamil dose–response curves of the ERGF and ERGS components; IC_{50} values were 2.3 ± 0.2 and $19.2 \pm 2 \mu\text{M}$, respectively, and the Hill coefficients were 0.94 and 1.44, respectively. *Inset*, Superimposed recordings of the ERGF tail currents at -100 mV under control conditions (line) and during $3 \mu\text{M}$ verapamil perfusion (○); the latter trace was multiplied by 2.1. The procedure used to isolate ERGF followed that shown in **C**. **E**, Recordings elicited by the protocol shown in **H**, *inset*. **F**, Traces after 10 sec conditioning at -100 mV in the same cell. **G**, Superimposed traces obtained after subtracting the data in **F** from those in **E**. **H**, The traces after perfusion of $3 \mu\text{M}$ verapamil (+ verap) in the same cell. **I**, Superimposed traces obtained after subtracting the data in **H** from those in **E**.

succeeding development of the deactivation process that is more or less complete and quick at different test potentials.

In MMQ cells (Fig. 1*A*), the long-lasting component (■) can be completely deactivated by 30 sec conditioning to -100 mV (●); both components are blocked by WAY 123,398 (▲). However, verapamil, which is a good blocker of ERG K^+ currents (I_{ERG}) (Chouabe et al., 1998; Zhang et al., 1999), did not block the long-lasting component (Fig. 1*B*, ●) at a concentration of $3 \mu\text{M}$ (*D*). As shown in Figure 1*B*, subtraction of the current obtained in verapamil from the control current leads to a trace that has the typical time course of ERG currents (Fig. 1*B*, ■–●), which we called ERGF. This suggests that the long-lasting component is a novel pharmacologically ERG-related K^+ current, which we called ERGS (ERG slow). Figure 1*C* (the same cell as that shown in Fig. 1*B*) shows that the difference between the control current (▲) and the current elicited after the -100 mV conditioning (▼), returns a component (ERGS, ▼–▲) with properties that are completely different from those of I_{ERG} . A trace very similar to that shown in Figure 1*C* (▼–▲) can be obtained in Figure 1*B* by subtracting a suitable amount of ERGF from the trace in verapamil (●) (Fig. 1, see legend). To investigate the reliability of this approach, we derived the verapamil dose–response relationships for the two current components in Figure 1*D*. They appear to be well separated: the IC_{50} of the ERGF was 2.3 ± 0.2 and that of ERGS was $19.2 \pm 2 \mu\text{M}$ ($n = 5$). Verapamil ($3 \mu\text{M}$) had negligible effects (Chouabe et al., 1998) on the activation curve of ERGF (shift of -3.1 ± 0.6 mV; $n = 4$; data not shown) and its deactivation kinetics (Fig. 1*D*, *inset*).

To study the voltage-dependent properties of the MMQ currents (Fig. 1*E–I*), we used the protocol shown in the *inset* to **H**; the control traces are shown in Figure 1*E*. A 10 sec hyperpolarization to -100 mV depressed the ERGS component (**F**), and this difference (**E**, **F**) produced the traces shown in **G**. When the control cell (**E**) was directly perfused with $3 \mu\text{M}$ verapamil (**H**), the resulting verapamil-sensitive currents (**I**) were very similar to

typical ERG currents (Arcangeli et al., 1995; Faravelli et al., 1996; Barros et al., 1997; S. Wang et al., 1997; Schönherr et al., 1999). In conclusion, the verapamil results suggest the coexistence of two components, ERGF and ERGS, which can also be isolated biophysically by means of ERGS deactivation (at -100 mV).

The voltage dependence of deactivation and recovery from inactivation

Because the most evident property of I_{ERGS} is its very slow deactivation, we studied this process in detail. The experimental protocol (Fig. 2*A*) consisted of two consecutive identical episodes elicited from a V_H of 0 mV at suitable test potentials (ranging from -60 to -120 mV and lasting for between 240 and 12 sec) in different trials; the membrane potential between the two episodes was brought to 0 mV by means of a slow ramp to reduce contamination from delayed rectifier currents before eliciting the second episode. During the first episode, the total inward current is elicited (first epi) and completely deactivated. The time at 0 mV was sufficient to reactivate only the ERGF component, and the second elicited current (second epi) is simply I_{ERGF} . This is shown in Figure 2*B* for membrane potentials ranging from -60 to -120 mV. In each of the panels, the trace corresponding to the difference between the first and the second episode is also shown, and we assume that it represents the isolated ERGS component (notice the different time scales). This trace is shown in Figure 2*C* at much slower time scales to highlight the complete deactivation process. An idea of the dramatic difference in the complete time course of the deactivation process for the two components can be seen in the two superimposed records in the last panel but one. The last panel in **C** shows the superimposed traces at different membrane potentials. The inset represents the theoretical reconstruction according to the model described in Materials and Methods.

The ERGS and ERGF traces were fitted to double exponential decaying functions. The time constants (τ_1^{ERGS} , τ_2^{ERGS} , τ_1^{ERGF} , τ_2^{ERGF}) and the fractional ratio of the associated amplitudes

Figure 2. Voltage- and time-dependent deactivation of the ERGS component. *A*, The protocol used for eliciting the deactivation recordings shown in *B* and *C*. The slow ramps to reach the holding level after the long pulses were chosen to reduce the activation of the delayed rectifier currents. *B*, The panels from left to right correspond to experiments done at -60 , -80 , -100 , and -120 mV. The appropriate time scale for each was chosen to show the ERGF (*1st epi*) and the ERGS (*2nd epi* – *1st epi*) components. Note particularly the very slow recovery from inactivation of the ERGS component in comparison with ERGF. The traces in each panel show the first and second episodes (WAY-corrected) and the difference between the two. *C*, The panels show (same experiments shown in *B*) only ERGS at much longer time scales to illustrate the complete process of deactivation. In the next to last panel, the second episode taken from the corresponding *B* panel is superimposed as indicated. In the last panel on the right, the traces elicited at -60 , -80 , and -100 are superimposed on that elicited at -120 mV. Inset, The traces were obtained using the computer model for ERGS (see Materials and Methods and Results).

Table 1. Deactivation time constant data ($\tau_{1\text{ERGS}}$, $\tau_{2\text{ERGS}}$, $\tau_{1\text{ERGF}}$, $\tau_{2\text{ERGF}}$; $n = 6$)

V_M (mV)	$\tau_{1\text{ERGS}}$ SEC	$\tau_{2\text{ERGS}}$ SEC	$A_{1\text{ERGS}}/(A_{1\text{ERGS}} + A_{2\text{ERGS}})$	$\tau_{1\text{ERGF}}$ msec	$\tau_{2\text{ERGF}}$ msec	$A_{1\text{ERGF}}/(A_{1\text{ERGF}} + A_{2\text{ERGF}})$	$\tau_{\text{rec-ERGS}}$ msec	$\tau_{\text{rec-ERGF}}$ msec
-60	4.2 ± 0.9	50.5 ± 5.6	0.54 ± 0.04	95 ± 12	1009 ± 300	0.81 ± 0.06	203 ± 23	11.2 ± 1.3
-80	3.6 ± 0.9	25.6 ± 8.1	0.67 ± 0.05	64 ± 15	550 ± 150	0.73 ± 0.03	130 ± 13	7 ± 1.1
-100	1.2 ± 0.2	6.5 ± 3.7	0.52 ± 0.1	23 ± 5	220 ± 70	0.85 ± 0.01	24 ± 5.2	3.9 ± 0.9
-120	0.2 ± 0.1	1.8 ± 0.2	0.44 ± 0.03	13 ± 4	120 ± 20	0.9 ± 0.015	12.6 ± 2.1	1.8 ± 0.5

Time constant of recovery from inactivation ($\tau_{\text{rec-ERGS}}$, $\tau_{\text{rec-ERGF}}$; $n = 10$).

($A_{1\text{ERGS}}/[A_{1\text{ERGS}} + A_{2\text{ERGS}}]$, $A_{1\text{ERGF}}/[A_{1\text{ERGF}} + A_{2\text{ERGF}}]$) were computed from six similar experiments, the averaged data of which are shown in Table 1. These recordings (the inward-going traces up to the peak current) were used to derive the time constants of the recovery from inactivation of ERGF and ERGS at the same membrane potentials (last two columns in Table 1). To calculate these time constants, we also used experiments such as those shown in Figure 1E–G ($n = 4$).

Because MMQ cells have a large inactivating delayed rectifier current, to evaluate the inactivation curve of ERGS we used the procedure of Smith et al. (1996) and Faravelli et al. (1996) on the normalized peak conductances from the data shown in Figures 1H and 2, B and C. The $V_{1/2}$ and slope of the Boltzmann fitting curves were -74.2 ± 3.4 and 12.7 ± 2.7 mV, which were different from those derived from the ERGF current data (-48.1 ± 1.9 and 28.2 ± 2.1 mV): the ERGS value of $V_{1/2}$ was left-shifted by ~ 26 mV, and the slope was steeper.

These results indicate that the voltage-dependent processes governing the ERGS and ERGF components are different because there is no overlapping of the time constants derived from the two components. Because the fast component is dominant, the ERGF data agree with those of Snyders and Chaudhary (1996), Schledermann et al. (2001), and Zhou et al. (1998), although the last were derived at 37°C . However, ERGS deactivation

is a double exponential process that is complete at -60 mV, thus indicating that activation should start at more depolarized membrane potentials, as shown below.

I_{ERGS} activation: distribution of I_{ERGS} in MMQ cells

To investigate the voltage-dependent activation of I_{ERGS} , we estimated the original amount at different potentials by applying short pulses at -100 or -120 mV (pulses T0, T1, or Tn) (Fig. 3A). At the beginning of each experiment, the ERGS component was checked (test pulse T0) and then completely deactivated by pulses of either -80 or -100 mV lasting 100 or 15 sec, respectively (as suggested by the data shown in Table 1). The test pulses were always preceded by a 15 sec V_H at 0 mV, which was necessary to reactivate the ERGF (but not the ERGS) component and to check whether ERGS was deactivated. Furthermore, membrane potential was conditioned to different levels ranging from -60 to $+40$ mV for periods of time ranging from 15 to 720 sec. Additional test pulses (Tn) returned the amount of the consecutively activated ERGS component.

To exclude the possibility that the repetitive application of brief tail pulses might produce a small deactivation of ERGS current and perturb the pure voltage-dependent activation process, we tested the available I_{ERGS} at different times (i.e., 50, 100, and 400 sec), resetting the conditions each time by completely deactivat-

Figure 3. Voltage- and time-dependent activation of the ERGS component. **A**, The protocol used for eliciting the pulses for the tests ($T0$ – Tn , 400 msec at -100 mV or 140 msec at -120 mV), the deactivation of ERGS, and the conditioning levels of activation. **B**, The exponential time course of $I_{\text{ERGS}}(t)/I_{\text{ERGS}}(\text{max})$ ratio of the experiments shown in the insets at conditioning levels of $+40$ (Δ), 0 ($\square$), and -40 mV ($\diamond$); the three insets show the superimposed traces elicited (at -120 mV) in the same cell with the protocol shown in **A**, but at different conditioning levels, at the following times: $V_M = +40$ and 0 mV, 20, 60, and 270 sec; $V_M = -40$ mV, 60, 240, 480, and 720 sec. The traces in small circles represent the test made before deactivation ($T0$, see **A**). **C**, The exponential time course of $I_{\text{ERGS}}(t)/I_{\text{ERGS}}(\text{max})$ ratio of the experiments shown in **D** and **E** derive from cells belonging to categories 5 ($\bullet$) and 6 ($\blacksquare$) (Table 2) at a conditioning level of 0 mV. **D**, Superimposed traces elicited in a category 5 cell, with test pulses (at -100 mV) at the times corresponding to the symbols ($\bullet$) of the plot shown in **C**. **E**, Superimposed traces elicited in a category 6 cell, with test pulses (at -100 mV) at the times corresponding to the symbols ($\blacksquare$) of the plot shown in **C**.

ing I_{ERGS} . We concluded that four to five brief test pulses during the exponential activation process did not significantly affect the time-dependent development of activation.

Figure 3B shows a typical experiment used to study the activation time constants and steady-state activation at three V_M values. The insets show the traces from experiments performed at conditioning potentials of $+40$, 0 , and -40 mV, with the trace circles indicating the initial current ($T0$). The traces with the smallest long-lasting current represent the starting level ($T1$) of the activation process (just after deactivation). The intermediate traces were elicited at different times (Fig. 3B, Δ , $\square$, $\diamond$). The increasing amount of ERGS, normalized to the maximal level derived from the amplitudes observed before ERGS deactivation, was plotted for $V_M = +40$ (Δ), 0 ($\square$), and -40 mV ($\diamond$), and the data were fitted to exponential curves to obtain the activation time constants ($V_M = +40$ mV, $\tau_a = 120 \pm 6.3$ sec; 0 mV, $\tau_a = 114 \pm 4.5$ sec; -40 mV, $\tau_a = 325 \pm 16$ sec). Activation was complete at $+40$ and 0 mV, but the activation reached a maximal level of 0.31 at -40 mV, which suggests a $V_{1/2}$ activation that is more positive than -40 mV. Similar experiments ($n = 11$) were performed by scanning membrane potentials ranging from -60 to $+40$. The normalized activation data versus membrane potential were fitted to a Boltzmann curve characterized by the following $V_{1/2}$ and slope values ($n = 12$) of -37.2 ± 3.5 and 4.9 ± 1.1 mV, to be compared with the ERGF values of -33.7 ± 1.9 and 6.5 ± 1.5 ($n = 8$) typical of MMQ cells. The time constants obtained at $V_M = +40$ and 0 mV were pooled because the values were not significantly different (the average was 123 ± 9 sec), and those obtained at -20 and -40 mV were 173 ± 11 sec and 289 ± 32 sec, respectively ($n = 10$). For comparison, the I_{ERGF} values were 0.4 ± 0.08 , 2.8 ± 0.23 , and 9.8 ± 0.4 sec ($n = 10$).

The activation was quicker in the few cells ($\sim 11\%$) almost devoid of the ERGF component (Fig. 3D,E). It can be seen both from the superimposed traces and from the fractional

Table 2. MMQ cells ($n = 175$) subdivided into six categories according to their relative proportions of I_{ERGS} and I_{ERGF}

Category	$I_{\text{ERGS}}/(I_{\text{ERGF}} + I_{\text{ERGS}})$ (%)	Distribution (%) of MMQ cells
1	0–1	12.6 ($n = 22$)
2	2–20	13.1 ($n = 23$)
3	21–33	16.6 ($n = 29$)
4	34–50	46.3 ($n = 81$)
5	51–98	8.6 ($n = 15$)
6	99–100	2.9 ($n = 5$)

activation (plotted in **C**) that the time constants (22 ± 0.7 sec, $n = 3$; 11.2 ± 0.9 , $n = 5$) were much faster than in the more common cases in which ERGF is normally present. Because the reasons for this difference are still unclear, these cells ($\sim 11\%$; see below) were excluded from the analysis described above.

This observation suggested that the ERGS fraction in relation to the total current in MMQ cells should be analyzed. By examining the ERGS amplitude at 400 msec (when ERGF is almost completely deactivated), it is possible to classify the cells on the basis of the percentage of I_{ERGS} present (Fig. 2). On the contrary, after ERGS is completely deactivated, the peak of the transient current represents the amount of the uncontaminated ERGF component. We therefore classified the cells into six categories on the basis of the percentage of I_{ERGS} versus the sum of I_{ERGF} and I_{ERGS} . The cells were categorized as shown in Table 2, and it can be concluded that $\sim 42\%$ of the cells have a low expression of ERGS component [$I_{\text{ERGS}}/(I_{\text{ERGF}} + I_{\text{ERGS}})$ ratio $< 34\%$]. This observation, as we will see during the current-clamp experiments, will have some relevance for the suggested physiological role of the ERGS component.

Figure 4. Effects of scorpion toxin ErgTx-2 on the long-lasting ERGS component. *A–D*, The superimposed currents elicited with the protocol shown below before (*A*), during the application of the toxin (10 μg/ml) (*B*), after 15 sec of washout (*C*), and after 60 sec hyperpolarization to −100 mV without toxin (*D*). Data in *A–D* are from the same cell. *E*, *F*, Superimposed recordings elicited with the protocol shown below before (■), during the 30 sec at −100 mV (□), which deactivated I_{ERGS} , after 600 sec holding at 0 mV (●, which shows that I_{ERGS} was reactivated), and during the application of ErgTx-2 (○, 30 sec at −100 mV). Data in *E* and *F* are from the same cell. *G*, Dose–response curve of the fractional I_{ERGF} or I_{ERGS} blocked as a function of ErgTx-2 concentration. The procedure used to isolate ERGF followed that shown in Figure 1C. The data were fitted by using the following equation: fraction of blocked current = $(1 + ([\text{ErgTx-2}]/\text{IC}_{50})^p)^{-1}$, with an IC_{50} of 2.02 ± 0.2 μg/ml ($n = 4$) and $p = 1.64 \pm 0.2$ for ERGS and 1.44 ± 0.2 μg/ml ($n = 4$) and $p = 2.5 \pm 0.9$ for the ERGF component, but the maximal block for this component was 0.19 ± 0.02 . *Inset*, The effect of ErgTx-2 on the ERGF component is shown in the three traces corresponding to control (line), 10 μg/ml ErgTx-2 (○), and 1 μM WAY 123,398 (line).

The specific action of a scorpion toxin on ERGS deactivation

Because anti-arrhythmic blockers (Schäfer et al., 1999) (data not shown) and the scorpion ErgTx toxin characterized by us (Gurrola et al., 1999; Scaloni et al., 2000) were equally effective on ERGS and ERGF, we looked for a peptide that could distinguish the two components and isolated a different toxin (ErgTx-2) from fraction II of the venom of the Mexican scorpion *C. noxius* Hoffmann (see Materials and Methods).

The effect of a single application of 10 μg/ml ErgTx-2 on the ERGS component was fast and completely reversible, as shown in Figure 4*A–C*. Figure 4*D* shows the effect obtained in the same cell after the inhibition of the ERGS component by means of prolonged hyperpolarization to −100 mV. Comparison of the traces in *B* and *D* indicates that the two modes of ERGS inhibition are not completely equivalent: the traces in *D* are more similar to typical ERG currents than those observed during toxin application. This behavior is still being studied in our laboratory, but it is worth noting that the drug modestly affects the peak current represented mainly by I_{ERGF} . Figure 4, *E* and *F*, shows the specific inhibition induced by the toxin on the very slow deactivation of ERGS recorded at −100 mV under control conditions and on the application of the drug. The experiment was performed according to the pulse protocol shown at the bottom of the panels. The current was tested briefly (■), the I_{ERGS} was recorded during deactivation (□) and retested after a 10 min recovery (●) to check that the reactivation was complete, and finally deactivation was recorded (○) during toxin application. Figure 4*F* shows the superimposed traces of the current before and during the action of the toxin, to be compared with the last panel but one in Figure 2*C*, which was obtained by the prolonged hyperpolarization to −100 mV.

Finally, Figure 4*G* shows the dose–response curve of the peptide for the two components, indicating an IC_{50} value of 2.02 ± 0.2 μg/ml for ERGS and 1.44 ± 0.2 μg/ml for ERGF. Because the ERGS/ERGF ratio of maximal block was 1:0.19, it can be concluded that the specific effect on the ERGF component is negligible [see the *inset* to *D* where the action of ErgTx-2 (10 μg/ml) and WAY (1 μM) are compared]. These experiments indicate that a peptide present in fraction II of a scorpion venom can be used to distinguish ERGS and the ERGF currents.

The effects of [K⁺]_o

The I_{ERG} dependence on [K⁺]_o has been studied extensively in cardiac cells (Shibasaki 1987; Sanguinetti et al., 1990), oocytes (Sanguinetti et al., 1995; Wang et al., 1997) and neuroblastoma cells (Arcangeli et al., 1995), and it has been found that the maximal conductance (open channel) has a strong dependence on [K⁺]_o at variance with respect to other K⁺ channel families and that the properties of the inactivation are right-shifted at high [K⁺]_o values (Wang et al., 1997). Because the two ERGF and ERGS components could belong to two different molecular structures, it should be feasible to find possible differences in this biophysical feature.

We measured I_{ERGS} in each cell at one or two [K⁺]_o values (in addition to [K⁺]_o = 40 mM) and then, after completely deactivating it, we measured the [K⁺]_o dependence of I_{ERGF} . Table 3 shows the $I_{\text{ERGF}}/I_{\text{ERGS}}$ ratio at different [K⁺]_o after the data were normalized to those obtained in our standard [K⁺]_o of 40 mM. If the [K⁺]_o dependence of the conductances of the two components were the same, we would have found a normalized ratio of 1.0 for all of the external [K⁺]_o values, but the relatively large change in this ratio (by a factor of two in the range from 10 to 80 mM) suggests that the two currents have different conductance

Table 3. The $I_{\text{ERGF}}/I_{\text{ERGS}}$ ratio calculated at the various values of $[K^+]_o$, normalized to the same ratio at $[K^+]_o = 40$ mM

$[K^+]_o$ (mM)	$(I_{\text{ERGF}}/I_{\text{ERGS}})_{[K^+]_o} / (I_{\text{ERGF}}/I_{\text{ERGS}})_{[K^+]_o = 40}$
10	1.43 ± 0.15 ($n = 5$)
15	1.31 ± 0.08 ($n = 5$)
20	1.14 ± 0.09 ($n = 5$)
40	1
80	0.76 ± 0.03 ($n = 5$)

The data derive from tail currents elicited at -100 mV, from $V_H = 0$.

properties or that their dependence of the inactivation curve on $[K^+]_o$ is different (Wang et al., 1997), or both. Because all the experiments were done at very negative potentials where the inactivation is nearly at its maximal value (~ 1), we think that the putative shift of the inactivation curve should be negligible. Therefore this result should be added to those previously found to be useful for characterizing ERGF and ERGS.

Properties of action potentials and firing in MMQ cells

Because the firing properties of MMQ cells have not yet been investigated, we studied them in the light of the role of the ERGF and ERGS K^+ currents. To characterize the diversity of the APs, we measured the maximum peak level (V_{PEAK}), plateau potential (V_{PLATEAU}), afterhyperpolarization (AHP), duration (t_D), and instantaneous frequency (F_i) calculated as the reciprocal of the interspike interval. The peak was usually well beyond $+10$ mV, and the V_{PLATEAU} was approximately -4 mV. The AHP was in the range -60 to -46 mV, and t_D and F_i showed the largest scatter: when the duration was below 100 msec, the plateau was absent (Fig. 5D). There are examples of a duration comparable with that typically observed in the heart, but much larger durations were also common. F_i is rarely constant, and large differences are possible in the 0.01–1 Hz range. A summary of the average characterizing parameters under control conditions is listed in Table 4.

Although it reduced the AP peak, TTX ($0.6 \mu\text{M}$) did not block firing, but nickel ($50 \mu\text{M}$) and nifedipine ($5 \mu\text{M}$) always inhibited firing, thus suggesting that the APs in MMQ cells are mainly sustained by Ca^{2+} currents; consistently, BAYK ($1 \mu\text{M}$), a dihydropyridine that increases the L-type Ca^{2+} channel open duration, considerably prolonged AP duration. The typical properties of single spontaneous APs under perforated-patch conditions are shown in Figure 5 (*insets*). Dopamine, the physiological neurotransmitter that inhibits prolactin release, induced a marked hyperpolarization that was sufficient to stop the firing (Fig. 5A); this effect was observed in $\sim 50\%$ of the cells ($n = 35$).

On the whole, these data suggest that most of the MMQ cells have the typical properties of native lactotrophs, which are known to be usually as spontaneously active as ours (Sankaranarayanan and Simasko, 1998). As discussed below, this explains the presence of a small amount of background prolactin release (Meucci et al., 1992).

Action of ERG/ERGS-blockers

Various effects were observed in 23 of 35 MMQ cells (66%) that were current clamped by using the perforated-patch technique before and after the application of ERG channels blockers, the most consistent being a significant increase in firing frequency (Table 4, third column).

A sample of the similar effects obtained after perfusing the

cells with different ERG blockers is shown in Figure 5B–E. Figure 5B shows increased firing frequency accompanied by a small reduction in AP duration and AHP; Figure 5C shows increased firing frequency without any alteration in AP duration and the disappearance of AHP. Experiments shown in Figure 5, D and E, were quantitatively analyzed. Figure 5D shows a cell that was spontaneously firing at ~ 1 Hz. After WAY application, two variables were monitored: instantaneous frequency and AHP, plotted as a *white line* or *white circles*, respectively. WAY application led to a reversible threefold increase in frequency and a decrease in AHP (4 mV). The traces before and during the application of WAY are shown at fast time resolution in the Figure 5D (*inset*). The AP wave shape was not altered except for the interspike interval, which was more depolarized during drug application. The cell shown in Figure 5E fired spontaneously at a much slower frequency (0.05) than the cell shown in D; the effect of E-4031 affected both the firing and AHP, increasing AP frequency ($\square$) by approximately fourfold and decreasing AHP ($\circ$) by ~ 6 mV. The wave shape of the action potential was also marginally changed, and the AHP was mostly inhibited during drug application.

The effects of long hyperpolarizations to -100 mV or scorpion toxin ErgTx-2 on firing properties

We have shown above that there are two ways to inhibit I_{ERGS} : (1) a long hyperpolarization to -100 mV and (2) the application of ErgTx-2 toxin. These methods should have different effects when applied to firing cells: the first deactivates I_{ERGS} by clamping the cell to -100 mV, and the subsequent restoration of V_{REST} returns a momentarily I_{ERGS} -free cell that will presumably fire at a higher frequency and thus progressively reactivate I_{ERGS} itself; the second should alter cell firing in a way that is reversible by washout.

We recorded the effect of a 40–50 sec hyperpolarization to -100 mV (obtained by means of suitable current injection) in 10 cells firing regularly at rest. Five of these cells showed a sudden increase in firing frequency after the offset of the hyperpolarization. The data from two successful cells are shown in Figure 6, A and B, and an exemplary nonresponding cell is shown in C. The plot of instantaneous frequency ($\circ$; the line is a running average) in A and B shows a net change in frequency change after the conditioning, and a subsequent slow decline to the original frequency (*straight line*).

The effect of blocking I_{ERGS} by ErgTx-2 was observed in five experiments (of eight), the results of two of which are shown in Figure 6, D and E; one unsuccessful experiment is shown in F. The toxin increased firing frequency from ~ 0.08 Hz under control conditions to 0.22 Hz during drug administration in the first cell (D) and from almost zero to 0.35 Hz in the second cell (E). There was no change in AP duration or AHP. In conclusion, the very low firing frequency of the cells in which these effects were observed again suggests and confirms that the specific role of I_{ERGS} is to produce adaptation at such low-frequency firing.

Interestingly, WAY application was ineffective in four of the five unsuccessful experiments based on the first method and in all three unsuccessful experiments based on the second method. It can be argued that when I_{ERGS} expression is too small to affect MMQ cell firing, I_{ERG} is unable to substitute it because it is unable to produce adaptation of the firing at very slow frequencies.

In conclusion, all of these experiments investigating the specific functional role of the ERGS component indicate that I_{ERGS}

Figure 5. Perforated-patch recordings of exemplary firing of MMQ cells and the effects of blockers. *A*, The result of the application of 5 μ M dopamine. *B*, WAY (way) affected firing frequency and reduced AP duration. *C*, E-4031 induced a marked reduction in AHP and an increase in firing frequency. *D*, Spontaneous firing of an MMQ cell before, during, and after WAY application. The plots of the instantaneous firing frequency (left scale, continuous line) and AHP (right scale, $\circ$) are superimposed in white. *E*, Spontaneous firing of a cell before, during, and after E-4031 application. The plots of the instantaneous firing frequency (left scale, $\square$) and AHP (right scale, $\circ$; data points were decimated) are superimposed. Insets in *A–C* show the wave shape of the APs indicated by the arrows. The insets in *D* and *E* compare the superimposed and aligned traces before and after drug application from the APs indicated by the arrows.

Table 4. Physiological parameters characterizing APs in MMQ cells under control conditions ($n = 35$; the first two columns)

Parameter	Control $n = 35$		Antiarrhythmics mV or %	ErgTx-2 ($n = 5$ of 8) mV or %	I_{ERGS} deactivation ($n = 5$ of 10) % change
	Average $\pm$ SE	Min/max			
V_{REST} (mV)	-45 ± 2	$-55/-38$	$+3 \pm 2.2$ mV, $n = 6$	4.1 ± 2.2 mV	$+2.8 \pm 1.9$ mV
V_{PEAK} (mV)	$+23 \pm 3.4$	$+40/+8$	-10.1 ± 2.9 mV, $n = 5$	ND	ND
V_{PLATEAU} (mV)	-4.4 ± 1.7	$-14/+5$	-4.1 ± 1.9 mV, $n = 6$	ND	ND
AHP (mV)	-51.7 ± 1.1	$-60/-46$	-4.3 ± 0.5 mV, $n = 9$	ND	ND
τ_D (msec)	2156 ± 832	80/9000	$-4.9 \pm 1.3\%$, $n = 2$	ND	ND
F_i (Hz)	0.2 ± 0.29	0.01/1	$+226 \pm 13\%$, $n = 25$	$+192 \pm 29\%$	$+245 \pm 35\%$

The observed changes (mean $\pm$ SE, n) in some of the parameters during perfusion with antiarrhythmics or ErgTx-2 and after I_{ERGS} deactivation (30 sec at -100 mV) are shown in the last three columns in millivolts (mV) or as percentages (%) of the control values. ND, Not detected; Min/max, minimum/maximum.

sustains an accommodation process at low firing rates at which ERG action is ineffective.

The action of anti-arrhythmic blockers on prolactin secretion

The strong hyperexcitability observed in Figure 6 prompted us to verify whether this response is functionally related to prolactin release (Bauer et al., 1999). As explained in Materials and Methods, prolactin secretion was measured ($n = 8$) before and after perfusion with the blocker WAY-123,398 in three different conditions: $[K^+]_o = 5$ mM, which corresponds to control; $[K^+]_o = 5$ mM + 10 μ M WAY, which corresponds to a complete blockade of the ERG and ERGS components; and $[K^+]_o = 40$ mM, which corresponds to an almost fully depolarized condition. The results indicate that there is a measurable prolactin release of 2.05 ± 0.1 ng/ml under purely physiological conditions (5 mM $[K^+]_o$) (Meucci et al., 1992); furthermore, at 10 μ M WAY (6.91 ± 0.7 ng/ml) and 40 mM $[K^+]_o$ (5.12 ± 0.5 ng/ml), prolactin increased 3.37 and 2.5 times, respectively, in comparison with baseline. These data are in line with the effects on cell firing.

The presence of different *erg* genes

The expression of the three currently known *erg* genes was studied in MMQ cells using the RPA (Shi et al., 1997). Rat brain was chosen as the control for *erg1* and *erg3*, and rat retina was the control for *erg2* expression. As shown in Figure 7, all of the three *erg* genes are expressed in MMQ cells, with *erg1* being the most abundantly represented and *erg3* being expressed at very low levels: an *erg3*-protected band (Fig. 7C) can be detected only after 1 week of autoradiographic film exposure (Fig. 7C, inset). It is worth noting that the additional bands, for all of the *erg* genes tested and detectable in both control tissue and MMQ cells, may have been caused by alternative gene splicing.

Firing properties predicted by a model cell with ERG or ERGS channels

Using the procedures indicated in Materials and Methods, we investigated the effects of adding either I_{ERG} or I_{ERGS} to a model simulating spontaneous firing. The onset was forced from a membrane potential of -60 mV (at which ERG and ERGS channels are completely closed), and after 2 sec the holding current was removed and the cell was allowed to fire and adjust its firing rate toward steady-state equilibrium. The result of this 200

Figure 6. The effect of I_{ERGS} deactivation and the ErgTx-2 on the firing properties of MMQ cells. *A–C*, Recordings of firing before and after 40–60 sec hyperpolarization to -100 mV in three different cells. Instantaneous frequency is shown in the bottom part of each plot (circles); the straight and continuous lines before and after the onset of the hyperpolarization indicate the average frequencies during these periods. Notice that there is a marked, transient increase in firing frequency in *A* and *B* that is not evident in *C*. *D–F*, Firing observed before, during, and after the application of ErgTx-2 ($10 \mu\text{g/ml}$). Notice that there are no noticeable effects in *F*. The insets show the wave shape of the various APs during an 800 msec window.

Figure 7. Presence of *erg* transcripts in MMQ cells. RNA extracted from rat brain, rat retina, and MMQ cells was probed using the rat (*r*) *erg1*, *erg2*, and *erg3* clones. Rat brain RNA was used as a control for *erg1* and *erg3*, and rat retina RNA was used as a control for *erg2* expression. Rat cyclophilin (Ambion) was used as an internal control, and yeast t-RNA was used as a negative control for the probe self-protection bands. *A*, *erg1* [three-dimensional (3D) exposure]; *B*, *erg2* (1D exposure); *C*, *erg3* (1D exposure); inset, higher magnification of the area indicated in *C*, taken from an autoradiographic film exposed for 7 d. *cyc*, Cyclophilin.

sec simulation is shown in Figure 8*A*, which shows that the cell has an almost constant firing frequency of 0.16 Hz (Fig. 8*A*, right scale, $\square$). The small decrease in action potential peaks during the 200 sec period was caused by long-term changes from the non-steady-state onset.

When ERGF conductance was added (Fig. 8*B*), firing remained constant (but at the lower value of 0.09 Hz), and AHP increased from -60 to -67 mV. Conversely, when only ERGS conductance was added (Fig. 8*D*), firing started at 0.13 Hz, but after 200 sec it dropped at a frequency of 0.05 Hz, which means an accommodation of 2.6 times. These results mimic those observed in Figure 6, *A* and *B*, that were obtained under similar experimental conditions, and those summarized in Table 4 that show an average frequency increase of $\sim 200\%$ during drug or biophysical blockade of ERGS. The effect of adding both ERGF and ERGS components is shown in Figure 8*D* (superimposed, in small open circles).

To clarify the reasons behind the effects seen in Figure 8, *B* and *D*, we plotted (Fig. 8*C,E*) the I_{ERGF} and I_{ERGS} (continuous lines) responsible for the firing shown in *B* and *D*. The amplitude of I_{ERGF} remained practically constant throughout the 200 sec period, whereas I_{ERGS} increased over time (with brief negative peaks to zero).

An expanded portion of the last part (from 173 to 200 sec) of the tracings shown in Figure 8, *C* and *E* (left scale), is shown in *F* and *H*, together with the corresponding V_M (dotted line, right scale). Figure 8*F* shows that I_{ERGS} is zero at peak AP, whereas in *H* I_{ERGF} is almost zero during the spike interval and reaches a peak during AP repolarization, thus aiding the increase in AHP.

Because these currents depend on the product of activation and inactivation voltage- and time-dependent variables, these were also plotted (in Fig. 8*G* for ERGS and *I* for ERGF). Although ERGS inactivation (dotted line) follows the AP timing, the number of open ERGS channels (continuous line) is an almost steady-state variable; conversely, the inactivation and number of ERGF open channels both reflect the time course of the AP. Unlike during fast spiking (Chiesa et al., 1997; Schönherr et al., 1999), at

Figure 8. Recordings obtained from computer-simulated firing in a model cell (for details see Results and Materials and Methods). *A, B, D*, Firing obtained in a model cell without or with ERGF or ERGS channels as indicated in the panels. The right scale plots the instantaneous frequency ($\square$). Total time scale was 200 sec. For comparison, the superimposed trace in *D* ($\circ$) represents the firing in a cell model in which both ERGF and ERGS were present. *C, E*, Plot of I_{ERGF} and I_{ERGS} during the traces shown in *B* and *D*. *F*, Superimposed traces of V_M (dashed line) and I_{ERGS} (continuous line) corresponding to the last 17 sec of the simulation shown in *D*. *G*, Time course of the activation and inactivation variables for the ERGS component during the last 17 sec. *H*, Superimposed traces of V_M (dashed line) and I_{ERGF} (continuous line) corresponding to the last 17 sec of the simulation shown in *B*. *I*, Time course of the activation and inactivation variables for the ERGF component.

such a low firing frequency there is no accumulation of I_{ERGF} , whereas its repolarizing role is shown in Fig. 8*H* during the 1-sec-long AP. Although I_{ERGF} is much larger than I_{ERGS} during the AP, it becomes almost negligible during the interspike interval, which gives ERGF channels sufficient time to close. This is not true for ERGS channels, which are unable to close during the 20 sec of the interspike interval (Fig. 8*G*).

Our model helps to clarify the different roles of ERGF and ERGS channels during the very slow firing typical of lactotroph cells. The low firing rates are controlled by ERGS channels down to extremely low frequencies of the order of the reciprocal of the deactivation time constant (~ 100 – 150 sec at average cell resting potential).

DISCUSSION

The properties distinguishing I_{ERGS} from I_{ERGF}

The K^+ current in MMQ cells is similar to that originally observed in native rat lactotrophs by Sankaranarayanan and Simasko (1996) (interpreted as an M-like current) and more recently described by Schäfer et al. (1999). It consists of two components (I_{ERGF} and I_{ERGS}) variably distributed among the cell population, in agreement with the native cell data (Table 2). The two components can be distinguished in at least four ways. (1) An appropriate concentration of verapamil inhibits a large fraction of the fast-deactivating current without influencing ERGS (Fig. 1*A*). (2) Appropriate voltage-clamp experiments made it possible to study the ultra-slow processes of ERGS deactivation and activation that are kinetically distinct from those measurable for the ERGF component (Figs. 1–3, Table 1). (3) A pure peptide (ErgTx-2) obtained from the scorpion *C. noxi* Hoffmann venom selectively inhibits I_{ERGS} (Fig. 4). (4) The two components have a different $[K^+]_o$ dependency (Table 3).

The currents responsible for the two components share the structural motifs involved in channel interactions with antiarrhythmic agents, but all of their other properties (especially their voltage dependence) lead to the conclusion that they corre-

spond to two different molecular entities. For the sake of simplicity, we shall treat these components as different channels.

Firing properties in cells expressing ERG channels and novel putative roles of ERGS channels

It has been shown that ERG channel blockade or mutated ERG channels in different tissues lead to various effects, such as the prolongation of the heart action potential, the depolarization of neuroblastoma and glomus cells of the rabbit carotid body (Arcangeli et al., 1995; Faravelli et al., 1996; Bianchi et al., 1998; Overholt et al., 2000), an increase in neuronal firing (Chiesa et al., 1997) and GH₃/B₆ and GH₃ cells (Barros et al., 1997; Weinsberg et al., 1997), contractions in opossum esophageal circular smooth muscle (Akbarali et al., 1999), and increased firing and insulin secretion in human β -cells (Rosati et al., 2000). The functional roles of ERG currents can therefore be divided into three categories.

(1) Inactivated K^+ channels are rescued during the decaying phase of a plateau potential in cardiac AP. This is caused by recovery from inactivation (a fast process) and leads to the immediate availability of ERG channels that is useful for obtaining faster hyperpolarization of the membrane. The increase in the number of available channels overcompensates for the reduction in the driving force of K^+ ions and therefore produces a peak of outward current that rapidly repolarizes the AP, as shown in Figure 8*B, C, H* [also see Ono and Ito (1995)]. A pronounced increase in AHP can also be predicted (Fig. 8).

(2) In spontaneously firing cells, or cells that are induced to fire short (5–50 msec) APs at a higher rate than 2–3 Hz, only a few (5–10%) ERG channels are open/inactivated. The very slow deactivation of ERG channels at V_{REST} values of approximately -55 mV generates an accumulation of I_{ERG} that functions as a brake (Schönherr et al., 1999) and produces spike frequency accommodation (Chiesa et al., 1997; Selyanko et al., 1999; Overholt et al., 2000; Rosati et al., 2000).

(3) The overlap of the voltage-dependent activation and inactivation processes produces a window current that, in nonexcit-

able cells such as neuroblastomas, is responsible for the regulation of V_{REST} and its depolarization in the presence of a blocker (Arcangeli et al., 1995; Faravelli et al., 1996; Overholt et al., 2000).

Although these roles are distinct, it frequently happens that they inter-react according to the percentage of I_{ERG} in respect to the total K^+ currents. It is not always easy to distinguish which is more important. However, other potential contributions to these phenomena may come from other K^+ channels, and although we can confirm that apamin and charibdotoxin (blockers of $K_{\text{(Ca)}}$ channels) do not greatly affect the firing in MMQ cells, we cannot exclude other channels (Sankaranarayanan and Simasko, 1998).

In conclusion, we suggest that the functional role of I_{ERGS} in MMQ cells is centered on ultra-low-frequency firing accommodation. This is based on (1) the biophysical characterization of I_{ERGS} ; (2) the results of the hyperexcitability produced either by ErgTx-2 blockade or hyperpolarization-induced I_{ERGS} inhibition, and (3) the results of the reconstructed firing in the I_{ERGS} model cell. This can be clearly seen in Figure 8E where the very long time constants of I_{ERGS} activation/deactivation lead to the accumulation of open channels (Fig. 8G) during the I_{ERGS} growing phase, and it has been produced experimentally (Fig. 6A,B). Moreover, in the model, the simultaneous presence of ERGS and ERGF components (Fig. 8D, ○) indicates that specifically at very slow frequencies (see the end of the record) the presence of ERGF does not affect the firing.

Consistency of prediction data and the experimental effects of blockers

Given the result of the classification of MMQ cells (Table 2), which is at least qualitatively similar to that of native cells (Schäfer et al., 1999), it is clear that ERGF channels are present in ~97% of the cells. If only I_{ERGF} were important for firing accommodation, it could be expected that ERG blockers would have a hyperexcitable effect in almost all of the experiments, but we found only a 66% success rate. On the other hand, the computer-simulated MMQ cells showed that the efficacy of ERG channels in producing an accommodation at 0.2 Hz is limited to ~50% (Fig. 8A,B), to be compared with 160% (Fig. 8D) obtained by introducing ERGS channels. Moreover, the typical MMQ firing properties listed in Table 4 show that the average frequency is ~0.2 Hz, thus making it reasonable to suppose that the presence of ERG channels is scarcely effective at modulating the cell firing in a relevant fraction of the MMQ cells (Fig. 8B). By adding all of the cells belonging to category 4, 5 and 6, and ~50% of those of category 3, we obtain ~66%, which is consistent with the observed result.

The biophysical properties of the channels related to the *erg2* and *erg3* genes found in MMQ cells are incompatible with the properties of I_{ERGS}

RT-PCR experiments in normal rat lactotrophs (Schäfer et al., 1999) and in MMQ cells (Wimmers et al., 2001) have shown the presence of three and two (*erg1*, *erg2*) genes, respectively. Although our RPA molecular biology data also showed the presence of the *erg3* gene (at a much lower level), we can confirm the suggestions of Schäfer et al. (1999) that no combination of the expressed gene conductances explains the electrophysiological data in MMQ cells. The novel current component therefore has biophysical properties that cannot be explained simply by the present molecular biology data, and further exploration of the MMQ genome is necessary.

Putative coassembly of ERG monomer with other monomers

More recently, a large number of different mRNAs have been found in native pituitary and GH₃/B₆ cells (Wulfsen et al., 2000), and we cannot exclude the possibility that ERG monomers may interact with other unknown monomers to form particular ion channels with combined properties. Interestingly, it has been shown that in MMQ cells injected with a point-mutated *erg* construct (erg1G630S), a dominant-negative suppression of the endogenous current can be observed 8 hr after injection (Wimmers et al., 2001). This finding and the fact that both ErgTx-1 and the anti-arrhythmic drugs block completely the MMQ ERG-like currents without distinguishing between ERGF and ERGS suggests that probably the ion channels producing ERGS are built up with at least one ERGF subunit. Vice versa, the action of ErgTx-2, which seems to recognize only the ERGS fingerprint, suggests that the ERGS subunit, when present, can coassemble with ERGF-producing heterologous channels. It is also known that ERG can coassemble with small units such as MinK or MiRP1 (MinK-related peptide 1) to produce currents with different biophysical properties (McDonald et al., 1997; Abbott et al., 1999). Because our tests in MMQ cells (data not shown) with the M current blocker XE-331 (Wang et al., 1998) were unsuccessful, we can suggest that probably ERG monomers do not assemble with KCNQ2 and KCNQ3. On the other hand, it is known that M and ERG currents can apparently coexist independently in neuroblastoma cells (Meves et al., 1999; Selyanko et al., 1999). Experiments performed in MMQ cells with chromanol 293B (50 μM), a blocker of KCNQ1 and I_{Ks} currents (Busch et al., 1997), have also been unsuccessful (data not shown).

Concluding remarks

Measurements of the prolactin released in controls and during WAY blocker action demonstrate that the amount of released hormone is approximately as large as the hormone obtained with a prolonged and probably exhaustive high K^+ depolarization. This suggests that the prolactin released by the cells expressing I_{ERGS} is sufficiently large with respect to prolactin released by the 12–25% of cells devoid of I_{ERGS} (Table 2). In conclusion, to set their pacemaker at the desired frequency, lactotrophs are endowed with I_{ERGS} . This current gives the cells a negative feedback system with genuine voltage-dependent properties and thus is linked only marginally to other signaling pathways dependent on intracellular factors or $[\text{Ca}^{2+}]_i$ changes (Kwieciński and Hammond, 1998). The data of Schäfer et al. (1999) showing that the ERG-like current is sensitive to TRH in native lactotrophs indicate that the working point of this feedback system is physiologically subject to hypothalamic supervision.

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