tant p53 in NIH 3T3 cells detected with antibody PAb 421 but not PAb 240 (Fig. 4), lends support to this hypothesis.

An alternative model to explain our observations is that both wild-type and mutant p53 can activate or repress transcription in a promoter- and cell-type–specific manner. In one sense, our observations are reminiscent of the effect of the viral erb A oncogenic protein, which normally inhibits gene activation in animal cells but unexpectedly functions as a hormonally regulated transcriptional activator in *Saccharomyces cerevisiae* (*19*). The mutant p53 used in this study could be a transcriptional activator, whereas wild-type p53 could be a repressor in NIH 3T3 cells. In KNIH and SW13 cells, mutant p53 does not act as an activator but also does not inhibit activity as does wild-type p53, suggesting that it has lost its ability to act as a repressor of *MDR*1 transcriptional activation in these cell types.

It is now generally agreed that genetic alterations in oncogenes combined with functional loss of tumor suppressor genes may account for the onset and development of a number of human cancers, and that Ras and p53 mutations are clearly involved in malignant transformation (*20*). Our results, therefore, may provide the basis for a model describing the molecular events that are involved in the regulation of *MDR*1 gene expression during the progression of human cancers.

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GnRH-Induced Ca^{2+} Oscillations and Rhythmic Hyperpolarizations of Pituitary Gonadotropes

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Secretion of gonadotropic hormones from pituitary gonadotropes in response to gonadotropin-releasing hormone (GnRH) is essential for regulation of reproductive potential. Gonadotropes from male rats exhibited an unusual form of cellular excitation that resulted from periodic membrane hyperpolarization. GnRH induced an oscillatory release of intracellular Ca^{2+} via a guanosine triphosphate (GTP) binding protein–coupled phosphoinositide pathway and hyperpolarized the gonadotrope periodically by opening apamin-sensitive Ca^{2+}-activated K^+ (SK) channels. Each hyperpolarization was terminated by firing of a few action potentials that may result from removal of inactivation from voltage-gated Na^+ and Ca^{2+} channels.

PITUITARY GONADOTROPES SECRETE luteinizing hormone and follicle-stimulating hormone in response to pulses of the peptide GnRH in the pituitary portal circulation. The biochemical responses of gonadodotropes to GnRH include an increase in phosphoinositide (PI) turnover, a rise in the con- centration of intracellular free Ca^{2+}, and activation of protein kinase C (PKC) (*1–5*). But the electrophysiological events underlying the stimulation-secretion coupling are less well known because of the difficulty of identifying gonadotropes in the heterogeneous population of pituitary cells. In sheep, GnRH has been reported to induce no change of gonadotrope membrane potential or resistance (*6*). In rat, the initial phase of GnRH-induced secretion of luteinizing hormone is reported not to require extracellular Ca^{2+}, but the later phase (after 2 min) does and is diminished by dihydropyridine Ca^{2+} channel blockers (*7*). In a gonadotrope cell line, GnRH potentiates voltage-gated Ca^{2+} currents (*4*), possibly by a PKC-dependent mechanism (*8*). Using gigaseal recording techniques (*9*) on identified gonadotropes of the male rat (*10–12*), we now show that GnRH induces rhythmic release of Ca^{2+} from the inositol 1,4,5-trisphosphate (IP_3)–sensitive store and that Ca^{2+} entry via voltage-gated Ca^{2+} channels may occur during action poten-

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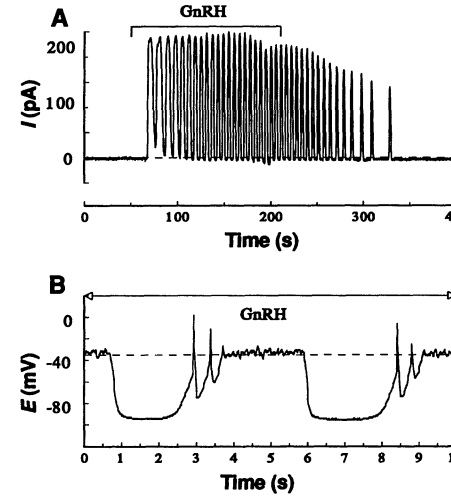

Fig. 1. Oscillations of membrane current and membrane voltage induced by GnRH. (**A**) Ionic currents (*I*) of a gonadotrope voltage clamped at −50 mV as GnRH solution (10 nM) was perfused into the bath during the 160-s period marked with a bar. In other cells exposed to GnRH continuously for 15 min, the rhythmic oscillations of outward current continued without decrement. (**B**) Membrane potential (*E*) changes of a gonadotrope continuously exposed to GnRH (100 nM) in the bath. Application started 3 min before the beginning of the trace. The dashed line denotes the resting potential (−35 mV) of this cell before GnRH was applied.

tials. Both mechanisms might contribute to GnRH-induced gonadotropic hormone secretion.

When gonadotropes were held under voltage clamp at −50 mV, application of GnRH (≥1 nM) consistently (157 of 164 cells) induced rhythmic outward membrane currents (Fig. 1A) with a cycle period ranging from 3 to 16 s (n = 87). This specific action at GnRH receptors was antagonized competitively by 10 to 1000 nM of the GnRH-antagonist peptide (*13*) D-pGlu[1], D-Phe[2],D-Trp[3,6] GnRH (n = 10). Rhythmic currents were evoked in cells held at any membrane potential between −140 and −10 mV; however, at potentials more negative than −100 mV, currents were inward.

We also examined the effect of GnRH on the membrane potential. Unstimulated gonadotropes rested at relatively depolarized potentials, −45 to −30 mV, and had little electrical activity. Addition of GnRH induced strong rhythmic hyperpolarizations (to −87 ± 5 mV; mean ± SD, n = 16), each of which was terminated by the firing of a few action potentials, and then quiescence at −37 ± 7 mV (Fig. 1B) (*11, 14*). Voltage clamp experiments show that gonadotropes, like other anterior pituitary cells, express a variety of voltage-gated Na^+, K^+, and Ca^{2+} channels (*15–17*). We find that the voltage-gated Na^+ and Ca^{2+} channels in these cells are half-inactivated at potentials of about −60 and −40 mV, respectively. Presumably, GnRH induces action potentials in these normally silent cells because each period of hyperpolarization transiently removes the resting inactivation of voltage-gated Na^+ channels and Ca^{2+} channels.

Evidence indicates that GnRH-induced current oscillations represent periodic openings of the SK type of Ca^{2+}-activated K^+ channels. First, apamin (0.4 to 1 μM), a selective blocker of SK channels (*18*), completely suppressed the current (n = 8) (Fig. 2A), whereas 1 and 5 mM tetraethyl ammonium (TEA) ion reduced the current by only 18 ± 5% and 53 ± 10% (n = 4, mean ± SEM) (Fig. 2A). Second, the oscillation is accompanied by increases of membrane conductance from 290 ± 80 pS at the minimum of the oscillation to 2700 ± 1600 pS (n = 12, mean ± SD) at the peak (Fig. 2B). Third, the conductance is K^+ selective, because the reversal potential follows the Nernst equation for K^+ ions as the bathing K^+ concentration is changed (Fig. 2C).

GnRH stimulates at least one pertussis toxin–insensitive guanosine triphosphate (GTP)–binding protein and PI turnover in various cells (*1, 4, 19, 20*). To test for involvement of GTP-binding proteins, we added guanine nucleotide analogues to the whole-cell pipette. Of eight cells exposed internally to guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S) (100 μM) for 5 min without exposure to GnRH, three showed one or two spontaneous increases in K^+ current and two developed a continuous, slow oscillation with a period of 25 s. A brief single exposure to GnRH irreversibly initiated oscillations (n = 12) (Fig. 3A), which continued for as long as 20 min. However, GnRH-induced responses could be prevented by including guanosine-5′-*O*-(2-thiodiphosphate) (GDP-β-S) (2 mM) in the pipette (four of four cells, 1 nM GnRH; three

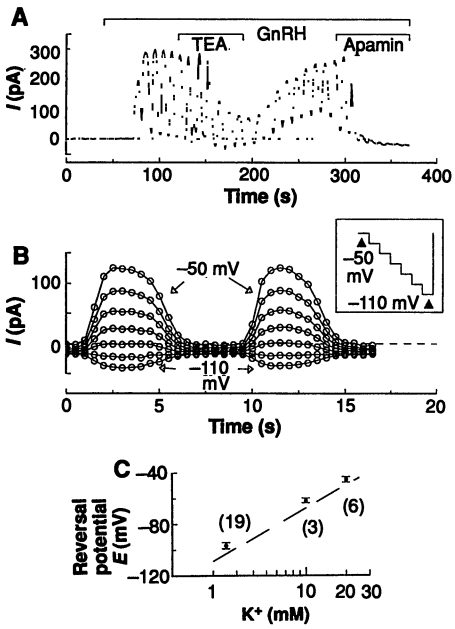

Fig. 2. Evidence that Ca^{2+}-activated K^+ channels carry the current induced by GnRH. (**A**) Effect of TEA (5 mM) and apamin (1 μM) on currents induced in a cell bathed with GnRH (10 nM). (**B**) Rapid measurement of membrane current-voltage relations during two cycles of oscillation in the continuous presence of 10 nM GnRH. A hyperpolarizing voltage waveform (inset), which consisted of a series of 10-ms voltage pulses descending by 10-mV steps from −50 to −110 mV, was applied every 0.5 s. Curves show mean current at each hyperpolarizing voltage as a function of time. At −100 and −110 mV, the induced current flowed inward. Membrane conductance values given in the text were calculated from the same data by determining the slope of plots of the mean current versus the voltage. (**C**) Reversal potentials for the induced current, measured as in (B), at a variety of external K^+ concentrations (K^+ replaced Na^+ in the standard bath solution). Symbols are mean ± SD with the number of determinations in parentheses. Dashed line is the predicted Nernst potential for K^+ ions at 25°C.

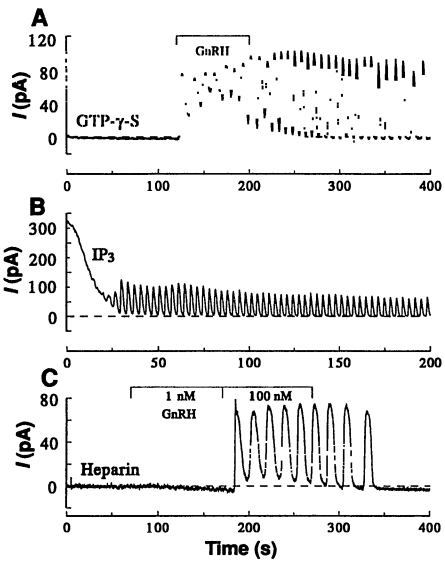

Fig. 3. Analysis of the intracellular signaling pathway. (**A**) Irreversible induction of oscillatory current by GnRH (10 nM) in a cell that had been exposed to a pipette solution containing GTP-γ-S (100 μM) for 10.5 min. (**B**) Response of a cell to the D isomer of IP_3 (20 μM) (LC Services Corp., Woburn, Massachusetts) in the whole-cell pipette. The trace begins 10 s after the patch was ruptured for whole-cell recording. In 20 cells, the Ca^{2+}-activated K^+ current was maximal (and even higher than the usual response to GnRH in other cells) at the beginning of the record, presumably because of a large initial Ca^{2+} release induced by the first exposure to IP_3. (**C**) Reduction of GnRH sensitivity by including heparin (1 mg/ml) (relative molecular mass, about 3000; Sigma Chemical Co., St. Louis, Missouri) in the whole-cell pipette. The record starts 4 min after the patch was ruptured. No response occurred with 1 nM GnRH, but a slow oscillation was induced by 100 nM GnRH.

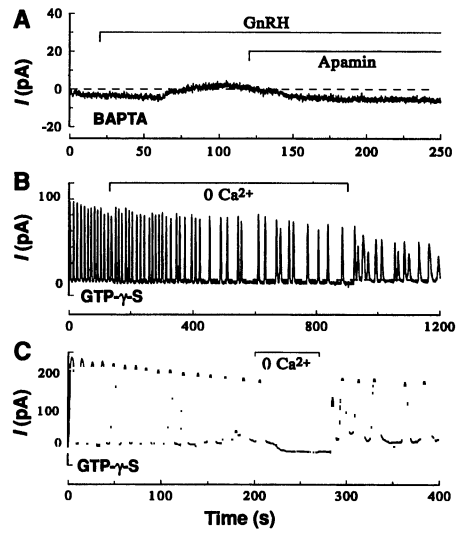

Fig. 4. Analysis of the calcium requirement. (**A**) Absence of oscillatory currents induced by GnRH when the whole-cell pipette included a calcium buffer with 120 nM free Ca^{2+} [5 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N′,N′*-tetracetic acid (BAPTA) and 2 mM Ca^{2+}]. The small outward current during application of GnRH (10 nM) was abolished by 100 nM apamin. (**B**) Gradual slowing of GnRH-induced oscillation when the normal bath solution was changed to a Ca^{2+}-free solution containing 6 mM Mg^{2+} and 1 mM EGTA. This cell was recorded with 100 μM GTP-γ-S in the pipette solution and was then activated irreversibly by a short exposure to GnRH (10 nM) 10 min before the beginning of the trace. (**C**) Example of reversible arrest of current oscillations during exposure to Ca^{2+}-free bath solution. We followed the same protocol as in (B) but used a different cell.

of four cells, 100 nM GnRH). Thus, these responses require a GTP-binding protein.

IP_3 also participates in the signaling pathway activated by GnRH. Persistent current oscillations were induced in the absence of GnRH when the pipette contained 16 to 20 μM of the D isomer of IP_3 (n = 20) (Fig. 3B), but not by 100 to 200 μM of the much less active (*21*) L isomer (n = 3). Cells dialyzed with the L isomer responded to subsequent GnRH stimulation normally. However, GnRH (1 nM) induced no current oscillation when the pipette contained the competitive IP_3-receptor antagonist (*22*) heparin (150 to 300 μM) (Fig. 3C); this block could be overcome with 100 nM GnRH (n = 6).

The opening of SK channels appeared to require a rise of the concentration of intracellular free Ca^{2+}, because GnRH induced minimal K^+ currents (n = 6) in cells exposed to an intracellular Ca^{2+} buffer solution (Fig. 4A). Consistent with the effect of IP_3, the Ca^{2+} seems to come from intracellular stores (*1, 3, 5, 16, 20*). When Ca^{2+} was omitted from the bathing solution, GnRH- or IP_3-induced current oscillations persisted at least 5 min in 8 of 11 cells (Fig. 4B). Nevertheless, even in these cells the amplitude and frequency of oscillations were reduced, and in the other three cells current oscillations stopped altogether (Fig. 4C). Presumably the intracellular Ca^{2+} stores eventually became depleted. Restoring extracellular Ca^{2+} partially reversed these effects.

These observations show electrophysiological activity induced by the natural stimulatory releasing hormone in identified anterior pituitary cells. Our results also indicate a possible new role for Ca^{2+} oscillations in the Ca^{2+} economy of the cell. Cyclical release of Ca^{2+} from intracellular stores may promote voltage-gated entry of extracellular Ca^{2+}, which could help to replenish the intracellular stores and promote hormone secretion.

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10. To dissociate cells, we incubated anterior pituitary glands of male (35 to 45 days) Sprague-Dawley rats with collagenase and trypsin [P. Thomas, A. Surprenant, W. Almers, *Neuron* **5**, 723 (1990)]. Gonadotropes were identified by means of the reverse hemolytic plaque assay (GnRH was used to stimulate secretion) (*11, 12*) with antibodies to luteinizing hormone (D. Leong, University of Virginia and American BioChem.) and then maintained in culture for 1 to 4 days before recording. All recording was done by whole-cell, gigaseal methods (*9*) with pipettes of 2.5- to 10-megohm series resistance at 20° to 25°C. Except in Fig. 1B, the membrane holding potential was set at −50 mV. Unless indicated, the bath solution contained 150 mM NaCl, 5 mM $CaCl_2$, 2.5 mM KCl, 1 mM $MgCl_2$, 8 mM glucose, and 10 mM Hepes (pH 7.4 with NaOH) and the pipette solution contained 120 mM potassium aspartate, 20 mM KCl, 0.1 mM GTP, 2 mM $MgCl_2$, 2 mM adenosine triphosphate (ATP), and 20 mM Hepes (pH 7.4 with NaOH). The junction potential (−10 mV) of this solution with the bath was corrected in all experiments. Except in Fig. 2C, no leak corrections were applied.
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Predicted Structural Similarities of the DNA Binding Domains of c-Myc and Endonuclease Eco RI

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The c-Myc oncoprotein belongs to a family of proteins whose DNA binding domains contain a basic region–helix-loop-helix (bHLH) motif. Systematic mutagenesis of c-Myc revealed that dimerized bHLH motifs formed a parallel four-helix bundle with the amino termini of helices 1 and 2 directed toward the inner and outer nucleotides of the DNA binding site, respectively. Both the basic region and the carboxyl-terminal end of the loop contributed to DNA binding specificity. The DNA binding domain of c-Myc may therefore be structurally similar to that of restriction endonuclease Eco RI.

A FAMILY OF DNA BINDING PROteins that includes c-Myc, E47, and TFEB is characterized by a conserved sequence referred to as the basic region–helix-loop-helix (bHLH) motif (*1*). The basic region of this motif contacts DNA, and the helices form a dimerization interface (*2*). Some of these proteins, such as c-Myc, have an additional dimerization motif called the leucine zipper (LZ) (*1, 3, 4*).

We sought to elucidate the tertiary structure of the c-Myc bHLH-LZ motif. As a first step, we defined the boundaries of its secondary structural elements (Fig. 1). Using the consensus amino acid sequence of bHLH proteins, we set the beginning of helix 1 at position 367 of c-Myc to demarcate the basic region from the downstream HLH motif. The end of helix 1 was set at position 378 of c-Myc since E12, E47, and TFEB each have a glycine corresponding to this position (*1, 4*) and glycines terminate one-third of the tabulated α-helices (*5*). The beginning of helix 2 for c-Myc was set at position 392, because the amino acids surrounding this position are not usually found at the NH_2-termini of α-helices (*5*). There are no helix-breaking residues between the beginning of helix 2 and the adjacent LZ, suggesting that these two elements may form a continuous α-helix.

DNA binding proteins that have a basic region fused to an LZ, such as the yeast transcription factor GCN4, dimerize with their LZs oriented in a parallel manner (*6*). To examine whether the LZs of bHLH-LZ proteins are also oriented parallelly, we substituted the LZ of a truncated form of c-Myc (MycPp) that contained only the bHLH-LZ domain (*7, 8*) with that of GCN4. The fusion protein (MycGL) bound DNA more efficiently than MycPp (Fig. 2), probably because the LZs of GCN4 homodimerize more readily than those of c-Myc (*9*). MycGL recognized the DNA binding site AC-CACGTGGT of c-Myc (*7, 10*). Altering the invariant (underlined) or inner nucleotides abolishes DNA binding by c-Myc (*7, 10*) and MycGL, while altering the outer nucleotides eliminates DNA binding by c-Myc (*7*), but

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