C3H10T1/2 cells in the absence of DNA synthesis (Fig. 3). Genistein inhibited the PDGF-induced, but not the TPA-induced phosphorylation of the 80-kD protein, confirming that its phosphorylation was not directly mediated by TPK activity (Fig. 3); rather, it resulted from indirect activation of PKC, probably through PIP_2 hydrolysis and DAG accumulation. PKC activity was not affected by genistein (Fig. 3, lanes 6 and 7). As with inositol phosphates and intracellular Ca^{2+}, replacement of the genistein- and PDGF-containing medium after 20 min or 4 hours with fresh 5% PDS-containing medium—without genistein and PDGF—did not result in PKC-dependent phosphorylation of the 80-kD protein, even after an additional 20-min incubation in the absence of genistein and PDGF (Fig. 3, lane 5). These results provide additional evidence that PLC was not activated by the down regulated PDGF receptor after genistein and PDGF removal, even though a full DNA synthetic response occurred. These results also support the conclusion of Coughlin *et al.* and others (*10*) that PKC activation alone is not sufficient for stimulation of DNA synthesis.

Genistein did not inhibit PDGF receptor down regulation (Table 2), but it did inhibit receptor autophosphorylation as shown by immunoblots of total cellular proteins with PDGF-induced phosphotyrosine (P-tyr) antibodies (Fig. 4). Several additional proteins were phosphorylated on tyrosine by the PDGF receptor; among them are proteins with sizes of 150 kD, 120 kD, 85 kD, and 75 kD. The identity of these P-tyr–containing proteins are currently unknown, but they have a similar molecular size to phospholipase C, $p21^{ras}$ GTPase-activating protein, phosphatidylinositol 3 (PI-3)–kinase, and $p74^{raf}$, respectively (*14, 15*).

Several candidates have been discussed as mediators of PDGF receptor action involving tyrosine phosphorylation. Several novel inositol phospholipids have been identified [phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate] that are phosphorylated on the 3 position of the inositol ring by a novel PI-3–kinase (*14*). The 85-kD PI-3–kinase has been shown to be associated with and phosphorylated by the PDGF receptor (*14*). In addition, $p21^{ras}$ GTPase-activating protein and $p74^{raf}$ are associated with the activated and autophosphorylated PDGF receptor (*15*). The functional significance of these PDGF receptor complexes remains to be determined, but on the basis of our findings, these associated proteins may be involved in the mechanisms by which PDGF induces DNA synthesis. Thus, with the exception of PDGF receptor down regulation, the early biochemical events of PIP_2 hydrolysis—accumulation of inositol phosphates, increase in intracellular Ca^{2+}, and PKC-dependent 80-kD protein phosphorylation—are not, as previously believed, necessary for PDGF-induced DNA synthesis. Our results indicate the potential importance of PDGF receptor down regulation in inducing competence and of the actions of the internalized PDGF receptor TPK activity in collaboration with associated proteins in promoting mitogenesis. We cannot eliminate the possibility that genistein is functioning apart from its inhibitory effects on TPK activity or is shunting the effects of PDGF into an alternate pathway that induces DNA synthesis. However, these reservations do not preclude the conclusion that these early biochemical events are not required for PDGF-induced DNA synthesis in fibroblasts.

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Endothelin Stimulation of Cytosolic Calcium and Gonadotropin Secretion in Anterior Pituitary Cells

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The presence of endothelin, a vasoconstrictor peptide, in the hypothalamus and posterior pituitary suggests that it also regulates neural and other nonvascular target cells. In pituitary gonadotrophs, low doses of endothelin evoked oscillations in the intracellular calcium concentration, and high doses induced a biphasic calcium response. Mobilization of intracellular calcium predominated during the spike phase of the calcium response to endothelin, whereas calcium entry through dihydropyridine-sensitive channels contributed to both the spike and plateau phases of the calcium response. Endothelin was as potent as hypothalamic gonadotropin-releasing hormone (GnRH) in stimulation of gonadotropin release in perifused pituitary cells. Endothelin bound specifically to pituitary cells with a dissociation constant of 70 picomolar, and induced rapid formation of inositol trisphosphate and diacylglycerol. Although intracellular calcium concentration and gonadotropin secretory responses to endothelin were independent of the GnRH receptor, endothelin and GnRH appeared to have a common signal transduction mechanism. These observations suggest that endothelin can act as a neuropeptide to regulate anterior pituitary function.

THE POTENT VASOCONSTRICTOR ACTION of endothelin (*1*) has been attributed to its promotion of calcium (Ca^{2+}) influx by acting directly on plasma membrane Ca^{2+} channels (*2*). However, like

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other vasoactive peptide hormones (angiotensin II, vasopressin), endothelin binds to specific receptors and activates phospholipase C to produce inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG) from phosphatidylinositol 4,5-bisphosphate (PIP_2) (*3*), leading to mobilization of intracellular Ca^{2+} and vascular smooth muscle contraction. Influx of extracellular Ca^{2+} through nonselective channels and sustained activation of dihydropyridine-sensitive Ca^{2+} channels prolongs the duration of the intracellular Ca^{2+} signal (*4*). In contrast to angiotensin II and vasopressin, endothelin action has been largely confined to the cardiovascular system. However, material that cross-reacted with antibodies to endothelin has been detected in the porcine spinal cord (*5*), as well as in the hypothalamus and posterior pituitary of pig and rat (*6*), suggesting that endothelin might also function as a neuropeptide. We now report that endothelin regulates Ca^{2+} and secretory responses in anterior pituitary cells, and acutely stimulates gonadotropin release with an efficacy comparable to that of endogenous hypothalamic GnRH.

When added to suspensions of rat pituitary cells, endothelin induced a biphasic pattern of changes in the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, with an immediate spike phase followed by a prolonged plateau phase (Fig. 1A). The maximum rise in $[Ca^{2+}]_i$ occurred within 10 s, and the duration of the spike phase was about 1.5 min. The magnitude of the plateau phase did not exceed 15% of the maximum response during the spike phase. The initial rapid increase of $[Ca^{2+}]_i$ occurred (with some reduction in amplitude) in the absence of extracellular Ca^{2+} (Fig. 1A), indicating that the spike phase resulted primarily from mobilization of intracellular Ca^{2+} stores.

The plateau phase was dependent on Ca^{2+} influx, and was rapidly terminated by addition of the dihydropyridine Ca^{2+} channel antagonist, nifedipine (Fig. 1B). Exposure to nifedipine attenuated the Ca^{2+} response to endothelin. The spike phase was reduced and the plateau phase was abolished (Fig. 1C), suggesting that entry of Ca^{2+} through L-type voltage-sensitive calcium channels (VSCC) represented the major calcium influx pathway in endothelin-stimulated cells. VSCC are present in all populations of hormone-secreting pituitary cells (*7*), and those in gonadotrophs undergo Ca^{2+}-dependent inactivation after stimulation by GnRH (*8*) or exposure to high extracellular potassium (*9*). In pituitary cells treated with depolarizing concentrations of potassium, endothelin still induced the spike phase of the Ca^{2+} response, but the functionally important plateau phase was abolished (Fig. 1D).

The amplitude of endothelin-induced Ca^{2+} response in cells from castrated animals (Fig. 1, A and D) was significantly higher than that of normal animals (Fig. 1, B and C). For castrated animals the change in $[Ca^{2+}]_i$ was 233 ± 22 nM ($n = 10$), while for normal animals it was 107 ± 9 nM ($n = 16$) ($P < 0.001$). This is consistent with the increased number of gonadotrophs in ovariectomized animals. Cytosolic Ca^{2+} imaging in single cells revealed that 25 to 30% of the anterior pituitary cells were sensitive to endothelin and their response to GnRH further confirmed that this endothelin-sensitive subpopulation was enriched in gonadotrophs (*10*). In single gonadotrophs, endothelin (100 nM) induced a biphasic elevation of $[Ca^{2+}]_i$ that was similar to the response elicited by GnRH (Fig. 2B). In contrast, low doses (1 nM) of both agents elicited an oscillatory pattern of $[Ca^{2+}]_i$ with decreasing amplitude during prolonged agonist stimulation. Addition of low concentrations of GnRH 2 min before endothelin stimulation had no effect on the increase in $[Ca^{2+}]_i$ (Fig. 2C), nor did low doses of endothelin affect the subsequent

Table 1. Inositol phosphate and diacylglycerol formation induced by endothelin in cultured pituitary cells from castrated animals. The cells were stimulated with 100 nM endothelin-1 for 20 sec. Values are expressed as mean ± standard error of the mean (SE). Ins 1,4,-P_2, inositol 1,4-bisphosphate; Ins 1,4,5-P_3, inositol 1,4,5-trisphosphate; Ins 1,3,4-P_3, inositol 1,3,4-trisphosphate.

	n	Ins 1,4-P_2 (cpm per 10^6 cells)	Ins 1,4,5-P_3 (cpm per 10^6 cells)	Ins 1,3,4-P_3 (cpm per 10^6 cells)	DAG (pmol per 10^6 cells)
Controls	5	408 ± 58	138 ± 24	0	89 ± 6
Endothelin	6	1422 ± 89*	303 ± 19*	162 ± 22	121 ± 8*

*$P < 0.05$, estimated by Student's *t* test.

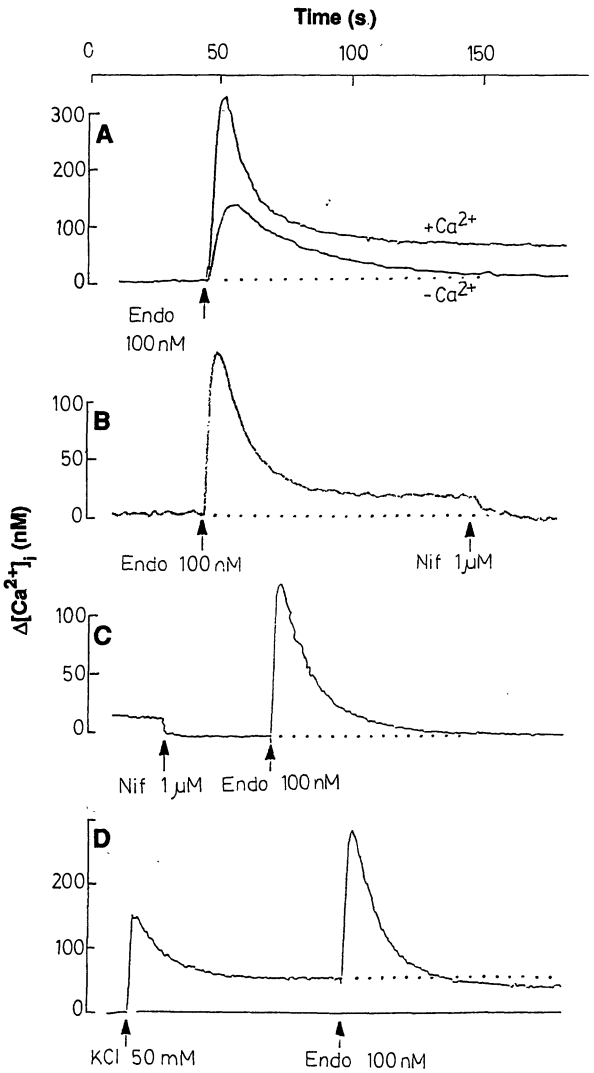

Fig. 1. Participation of calcium mobilization and entry in modulation of $[Ca^{2+}]_i$. Anterior pituitaries were removed from normal or castrated female Sprague-Dawley rats and dispersed into single cells by controlled trypsinization (*18*). The cells were incubated with 1 μ*M* Fura-2 AM for 30 min at 37°C. One to two million cells were used for $[Ca^{2+}]_i$ assay by fluorescence analysis (dual excitation wavelength; 340 and 390 nm in a 3-ml cuvette, Delta Scan Spectrofluorimeter, Photon Technology). The $[Ca^{2+}]_i$ values calculated from emission data at 500 nm were corrected for dye leakage and autofluorescence as described (*19*). (**A**) Addition of 100 nM endothelin-1 (Peninsula) to suspensions of pituitary cells from normal animals in the presence of 1.25 mM calcium ($+Ca^{2+}$), and in the absence of extracellular calcium ($-Ca^{2+}$). (**B**) Ca^{2+} response to endothelin (100 n*M*) after 60 s followed by addition of the calcium channel antagonist, nifedipine (Nif, 1 μM). (**C**) Ca^{2+} response in cells treated with Nif (1 μM) and subsequently with endothelin. (**D**) Ca^{2+} response to endothelin in cells treated with 50 mM potassium chloride (KCl). Results are representative of at least five experiments with cells from castrated (A and D) or normal animals (B and C). The peak value of $[Ca^{2+}]_i$ in the cells treated with Nif (C) was significantly lower than in controls (B) (controls 100 ± 6.3 compared to Nif-treated, 76.9 ± 7.5%, $P < 0.05$, estimated by Student's *t* test before normalization of data). Time scales are the same for all four panels.

GnRH response. However, prior addition of high concentrations of GnRH or endothelin inhibited mobilization of intracellular Ca^{2+} by subsequent addition of the other hormone (*11*), indicating a cross-desensitization of the cellular response.

The endothelin-induced rise in $[Ca^{2+}]_i$ is similar to the Ca^{2+} responses in many cell types that are regulated by activation of Ca^{2+} mobilizing receptors (*12*). In such cell types, a temporal correlation is observed between the rise in $[Ca^{2+}]_i$ and hormone release. To determine whether the prominent effects of endothelin on $[Ca^{2+}]_i$ are coupled to exocytosis in gonadotrophs, we analyzed its actions on the release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (*13*). Endothelin (100 n*M*) stimulated the secretion of both LH and FSH in perifused pituitary cells (Fig. 2A). The secretory profiles for both hormones closely followed the Ca^{2+} response, with an early peak and a sustained plateau phase. With a rapid perfusion system, we detected LH release 5 s after exposure to endothelin (Fig. 4B). Peak gonadotropin release occurred within 40 s, and the duration of the first phase of the secretory response was 1.5 min (Fig. 4B). Other pituitary hormones, including thyrotropin, growth hormone, and prolactin, exhibited relatively smaller responses to endothelin (*11*).

We observed a marked change in the Ca^{2+} response profile with rising endothelin doses (Fig. 3A). The spike and plateau phases of the Ca^{2+} response showed differential sensitivity to increasing doses of endothelin, suggesting that Ca^{2+} entry is more sensitive than Ca^{2+} mobilization to agonist stimulation. A similar dissociation at low agonist concentrations occurs in GnRH-stimulated gonadotrophs (*14*). Release of LH also showed a dose dependent increase with rising concentrations of endothelin (Fig. 3B). A comparison of the dose-related actions of endothelin on $[Ca^{2+}]_i$ and gonadotropin release revealed that the concentration of endothelin needed to achieve half-maximal stimulation for both parameters is about 8 nM (Fig. 3B).

Addition of a potent GnRH antagonist before or during agonist stimulation abolished the GnRH-induced rises in $[Ca^{2+}]_i$ and LH release (Fig. 4A), but did not affect the Ca^{2+} and LH responses to endothelin. The GnRH antagonist did not eliminate the endothelin-induced rise in $[Ca^{2+}]_i$ when added before or after endothelin (Fig. 4, C and D), and did not alter the LH secretory response to endothelin in perifused pituitary cells (Fig. 4B). ^{125}I-labeled endothelin bound specifically to rat pituitary cells with a dissociation constant (K_d) of 70 pM, and

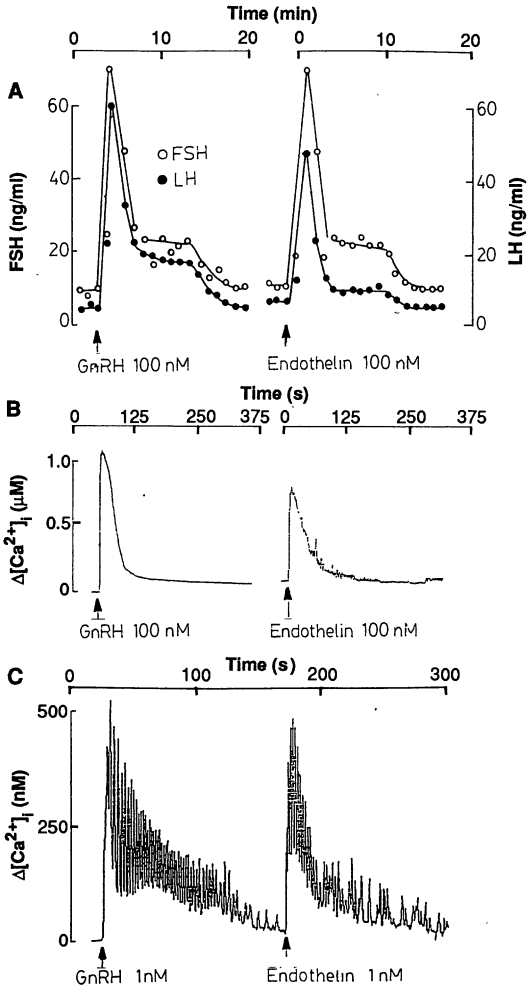

Fig. 2. Effects of GnRH and endothelin on Ca^{2+} and gonadotropin responses. Column perifusion was performed with 2×10^7 pituitary cells, from normal animals, that had been cultured for 3 days on Cytodex-1 beads (Pharmacia LKB Biotechnology). Cells on the beads were perifused with medium 199, 20 mM Hepes, and 0.1% bovine serum albumin for 2 hours (0.6 ml/min) and then stimulated with GnRH (Peninsula Lab, Inc.) or endothelin-1; fractions were collected at 1-min intervals. For $[Ca^{2+}]_i$ measurements, highly purified pituitary gonadotrophs from castrated animals were plated on 25-mm cover slips coated with poly-L-lysine, loaded with 2 μM Indo 1-AM for 60 min at 37°C, and mounted on the stage of an inverted Diaphot microscope attached to a Nikon intracellular Ca^{2+} analysis system. All $[Ca^{2+}]_i$ values were derived from a standard curve that was constructed by addition of various doses of Ca^{2+} to 10 μM Indo-1. (**A**) Release of LH and FSH at high doses of GnRH and endothelin. Data points are the means from four experiments and the values for standard error are within 10% of the means. (**B**) $[Ca^{2+}]_i$ in single gonadotrophs after equivalent doses of GnRH and endothelin. (**C**) Oscillations of $[Ca^{2+}]_i$ in the same gonadotroph after a low dose of GnRH and subsequent addition of endothelin.

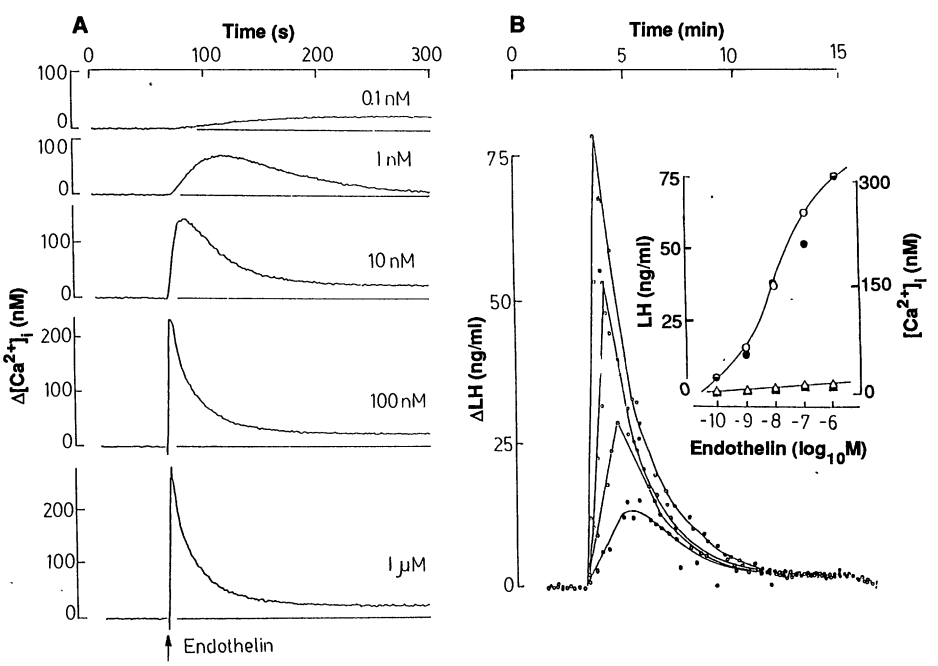

Fig. 3. Dose-dependent effects of endothelin on Ca^{2+} and gonadotropin responses. Experiments are performed on cells from castrated animals. (**A**) Ca^{2+} responses to endothelin in pituitary cells suspensions, in the doses indicated above the curves. (**B**) LH secretory response to endothelin in perifused pituitary cells. Curves from bottom to top: 1, 10, 100, and 1000 nM. (**Inset**) Comparison between the dose-dependent actions of endothelin on $[Ca^{2+}]_i$ and LH responses. Open symbols, $[Ca^{2+}]_i$; closed symbols, LH; circles, spike phase; triangles, plateau phase. Results shown are representative of three experiments.

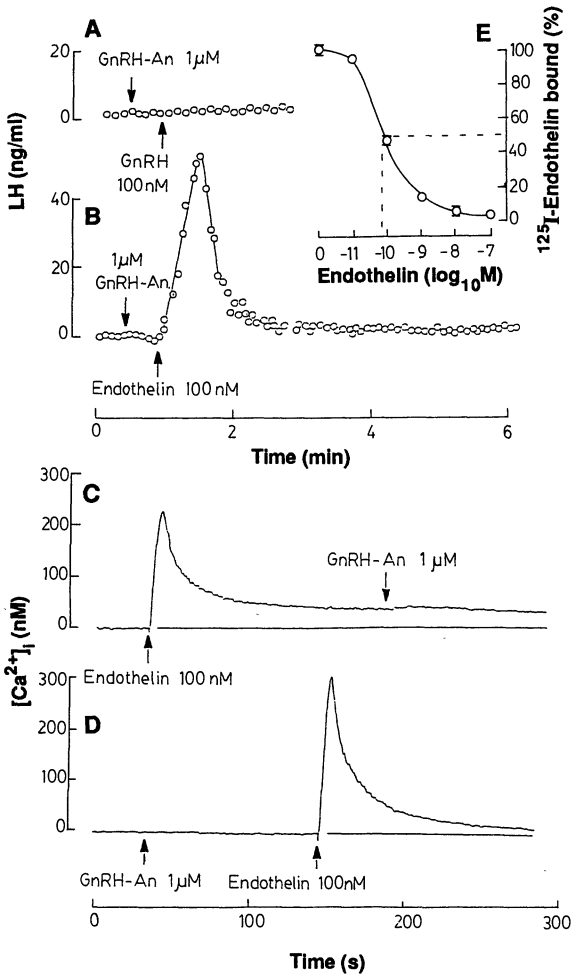

Fig. 4. Effects of a GnRH antagonist on GnRH- and endothelin-induced Ca^{2+} and LH responses. (**A**) Inhibition of GnRH-induced LH release in the presence of a GnRH antagonist, [*N*-acetyl-D-*p*-Cl-Phe[1,2], D-Trp[3], D-Lys[6], D-Ala[10]] GnRH (GnRHan). (**B**) Endothelin-induced LH release from cells in the presence of GnRHan. Endothelin-induced Ca^{2+} response in cells prior to (**C**) or followed by (**D**) addition of GnRHan. Time scale in (A) is as in (B); time scale for (C) is as in (D). (**E**) Dose-dependent inhibition of ^{125}I-labeled endothelin binding to pituitary cells from normal animals by unlabeled endothelin. ^{125}I-labeled endothelin binding studies were performed at 22°C for 120 min as described (*7*). Each point represents the mean ± SE of triplicate values, and the results are representative of three similar experiments. Scatchard analysis of data revealed a K_d of 70 pM.

unlabeled endothelin inhibited this binding in a concentration-dependent manner (Fig. 4E). In addition, endothelin induced rapid formation of both DAG and inositol phosphates (Table 1), indicating that the vasoconstrictor peptide stimulates phospholipase C–dependent hydrolysis of PIP_2 in anterior pituitary cells.

GnRH also acts through mobilization of Ca^{2+} from intracellular and extracellular sources to stimulate release of LH and FSH (*15*). This action is initiated by the phospholipase C–catalyzed hydrolysis of PIP_2, with rapid production of $InsP_3$ and diacylglycerol (*16*). The spike phase of the GnRH-induced Ca^{2+} response represents the primary signal for activation of the secretory response, while the entry of extracellular Ca^{2+} across the plasma membrane supports the sustained phase of secretion (*15*). Both dihydropyridine-sensitive and -insensitive pools of receptor-activated Ca^{2+} channels are believed to participate in GnRH-induced Ca^{2+} influx (*15*). We observed that endothelin initiated increases in $[Ca^{2+}]_i$ and gonadotropin release in a manner that was independent of the GnRH receptor; however, both peptides utilized the same second messenger system to elicit the secretory response.

Three other vasoactive hormones—angiotensin II, norepinephrine, and vasopressin—modulate secretion of certain pituitary hormones (*17*) but are weak or ineffective stimuli of gonadotropin secretion. In contrast, endothelin acutely released LH and FSH as efficiently as GnRH (Fig. 2A), suggesting that endothelin could contribute to the physiological control of gonadotropin secretion. Identification of cross reacting material to anti-endothelin in the supraoptic and paraventricular neurons, and in their terminals in the posterior pituitary gland (*6*) together with our results support the hypothesis that endothelin acts as a neuropeptide hormone. We do not yet know whether endothelin acts as a local mediator in anterior pituitary cells, or as a typical neurohormone through the hypothalamo-pituitary portal system.

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