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Calcium Signaling and Secretory Responses in Endothelin-Stimulated Anterior Pituitary Cells

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SUMMARY

Endothelin (ET) receptors are present in pituitary cells and stimulate hormone release through the phosphoinositide/Ca^{2+} signaling system. In pituitary cell suspensions, ET caused $[Ca^{2+}]_i$ elevations of much higher amplitudes than those induced by other vasoactive hormones, including angiotensin II, vasopressin, and noradrenalin. The action of ET was coupled to rapid and transient activation of exocytosis in gonadotrophs, thyrotrophs, somatotrophs, and lactotrophs. In contrast, angiotensin II did not stimulate luteinizing hormone release, and luteinizing hormone responses to vasopressin and noradrenalin were very small. Single gonadotrophs exhibited three types of $[Ca^{2+}]_i$ responses to increasing doses of ET, (a) subthreshold responses, with amplitude modulation; (b) threshold-oscillatory responses, with frequency modulation; and (c) threshold-biphasic responses, as the summation of single Ca^{2+} spikes. The same $[Ca^{2+}]_i$ patterns were also seen in gonadotropin-releasing hormone (GnRH)-stimulated cells. In the presence of $[Ca^{2+}]_e$, the amplitudes of the Ca^{2+} spikes progressively decreased during continuous stimulation with ET or GnRH, reaching the nonoscillatory plateau level after 200–400 sec of stimulation. In cells stimulated with GnRH, subsequent exposure to ET, GnRH, or ionomycin during the plateau phase did not elicit further increases in $[Ca^{2+}]_i$, whereas cells stimulated with ET responded partially to all three agents. In addition, cells exposed to ET or GnRH for 30 min, followed by a 30-min recovery period, were able to mount a full $[Ca^{2+}]_i$ response to GnRH, but not to ET-1. Similarly, both peptides elicited rapid increases in LH release, with comparable potencies, but the response to ET decreased much more rapidly during sustained stimulation and gonadotrophs became refractory to further ET stimulation. This is in part attributable to rapid endocytosis of ET receptors during continuous agonist stimulation. These data indicate that ET exerts potent but transient secretory actions in several pituitary cell types and is a potential regulator of gonadotropin release. The initial receptor-coupling events in both ET- and GnRH-stimulated cells are similar, but the differences observed during continuous or repetitive stimulation indicate that the ET receptor pathway undergoes rapid desensitization that is critical in determining the distinct cellular responses to the two peptides.

ET is a 21-residue vasoconstrictor peptide originally isolated from porcine aortic endothelial cells (1). The several ET-related peptides, ET-1, ET-2, and ET-3 (2), share structural and functional similarities with the SRTX S6b peptide isolated from the venom of the snake *Actractaspies engaddensis* (3). The ET peptides exert potent vasoconstrictive actions on a variety of blood vessels from many species (1). Because ET induces strong and sustained vasoconstriction, it is thought to contribute to local control of the circulation and blood pressure. Based on its homology with a group of calcium channel agonists and the observation that ET-induced contraction was inhibited by removal of extracellular Ca^{2+}, as well as by dihydropyridine calcium channel blockers, ET was originally proposed to induce contraction by stimulating Ca^{2+} influx through voltage-sensitive calcium channels (1). Subsequently, ET has been shown to stimulate release of Ca^{2+} from intracellular stores in a manner similar to that of typical calcium-mobilizing receptors (4). Further studies have confirmed that specific ET receptor binding is not competed for by calcium channel antagonists and that ET action on vascular smooth muscle involves inositol trisphosphate formation and calcium mobilization, as well as diacylglycerol accumulation and activation of protein kinase C (5–8).

The presence of ET in the brain (9) and neurohypophysis (10) and its direct *in vitro* effects on release of substance P (11), vasopressin (12), prolactin (13), and aldosterone (14) indicate that the peptide may be involved in neuroendocrine regulation. We have recently demonstrated that ET is a potent stimulus of LH and FSH from pituitary cells *in vitro*, with activity comparable to that of the physiological regulatory

ABBREVIATIONS: ET, endothelin; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; PRL, prolactin; GH, growth hormone; ACTH, adrenocorticotropin; TSH, thyrotropin; VP, Arg^8-vasopressin; AII, angiotensin II; $Ins(1,4,5)P_3$, inositol trisphosphate; $[Ca^{2+}]_i$, cytoplasmic calcium concentration; $[Ca^{2+}]_e$, extracellular calcium concentration; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

peptide GnRH. This action of ET is initiated by the activation of specific ET receptors that are coupled to phosphoinositide hydrolysis and Ca^{2+} mobilization (15). In the present paper, we have characterized the distribution of ET and other vasoactive peptide-induced responses within subpopulations of hormone-secreting pituitary cells. Such analyses have demonstrated that ET is the major vasoactive hormone with gonadotropin-releasing activity *in vitro*. In addition, Ca^{2+} signaling and secretory responses in ET-activated gonadotrophs showed complex patterns that were similar to GnRH-induced responses during the initiation of target cell activation, but not during continuous or repetitive stimulation of the gonadotroph.

Experimental Procedures

Measurement of secretory responses. Anterior pituitary glands from 2-week ovariectomized female rats were enzymatically dissociated, and the dispersed cells were cultured, as previously described (16), on preswollen Cytodex-1 beads (1.5×10^7 cells/250 μl of beads; Pharmacia, Piscataway, NJ). Columns of beads carrying 3-day cultured cells were perifused with warmed (37°) medium 199 (25 mM HEPES, 0.1% bovine serum albumin, and antibiotics) for 2 hr, at a flow rate of 0.6 ml/min. Fractions were collected every minute and stored at −20° before radioimmunoassay. For static cultures, dispersed cells were plated at 2.5×10^5 cells/well in 24-well tissue culture plates (Falcon, Oxnard, CA). After 3 days, the incubation medium was replaced by warmed HEPES-medium 199, containing selected concentrations of GnRH, ET, AII, VP (all from Peninsula Laboratory, Belmont, CA), or noradrenalin (Research Biochemicals Inc., Natick, MA). After incubation for 3 hr at 37°, in a water-saturated atmosphere of 5% CO_2 in air, media were removed and stored at −20° before radioimmunoassay, which used the LH, FSH, TSH, GH, PRL, and ACTH reagents and standards provided by the National Pituitary Agency (Baltimore, MD). Replicates of six wells were used in all experiments, and all samples for the experiments were analyzed in duplicate in the same assay.

Cytosolic calcium assays. Measurements of $[Ca^{2+}]_i$ in cell suspensions or in single gonadotrophs were performed as previously described (15, 16). Briefly, 2×10^7 dispersed cells preincubated in medium 199 at 37° for 60 min. Cell suspensions were loaded with fura-2 by incubation with 1 μM fura-2/acetoxy methyl ester (Calbiochem, San Diego, CA) for 30 min at 37°. Two million cells were used for $[Ca^{2+}]_i$ assay by fluorescence analysis, in a 3-ml cuvette in a Delta Scan spectrofluorimeter (Photon Technology, Inc.). For $[Ca^{2+}]_i$ measurements in single cells, 3-day-old pituitary cells plated on 25-mm coverslips coated with poly-L-lysine were loaded with 2 μM indo-1/acetoxy methyl ester (Calbiochem) for 60 min at 37° and mounted on the stage of an inverted Diaphot microscope attached to an intracellular calcium analysis system (Nikon, Inc., Melville, NY). All Ca^{2+} values were derived from a standard curve, which was constructed by addition of known concentrations of Ca^{2+} to 2 μM indo-1.

^{125}I-ET binding and internalization. Pituitary cells were plated into six-well Costar plates (4×10^6 cells/well). Three-day-old cultures were washed (2 × 2 ml) with medium 199 Hanks' solution, containing 25 mM HEPES, 0.1% bovine serum albumin, and penicilin/streptomycin. Dishes were kept at 4° during addition of ^{125}I-ET-1 or ET-2 (final concentration, 0.25 nM/well) without or with different doses of ET-1. The dishes were incubated at 37° for the appropriate time intervals. After incubation, the medium was removed and cell layers were washed (3 × 2 ml) with cold phosphate-buffered saline containing 0.2% bovine serum albumin, at 4°. To determine the surface-bound and internalized ^{125}I-ET, the cell monolayers were washed at 4° with 0.2 M acetic acid (pH 2.5) containing 0.5 M NaCl (2 × 10 min). The remaining cell-associated radioactivity was quantitated after solubilization of cells in 1 M NaOH containing 0.1% sodium dodecyl sulfate. Total cell-bound radioactivity was estimated in a parallel series of dishes by solubilization of cells without prior acid treatment. In some of the experiments, the internalized ^{125}I-ET was estimated by trypsinization, with similar results.

Statistical analysis. All results shown are representative of at least three different experiments. Where appropriate, the data are expressed as means ± standard errors. Student's t test was used for statistical comparison among means, and differences with $p < 0.01$ were considered significant.

Results

Effects of vasoconstrictor peptides on $[Ca^{2+}]_i$ and LH exocytosis. In the presence of extracellular Ca^{2+} (1.2 mM), ET-1 (100 nM) induced an initial rapid $[Ca^{2+}]_i$ peak in pituitary cells in suspension, followed by a second sustained phase (Fig. 1A, *upper trace*). When ET-1 was added to pituitary cells incubated in Ca^{2+}-deficient medium, the transient increase was attenuated and the sustained phase was abolished (data not shown), suggesting that receptor-mediated increases in $[Ca^{2+}]_i$ are initiated by $Ins(1,4,5)P_3$-induced release of Ca^{2+} from intracellular stores and are maintained by increased influx of Ca^{2+} through the plasma membrane. In agreement with this, the analysis of inositol phosphates produced in [^{3}H]inositol-labeled rat pituitary cells revealed extremely rapid and prominent increases of $Ins(1,4,5)P_3$ and inositol bisphosphate during stimulation with ET-1 (15). On the other hand, the ability of ET-1

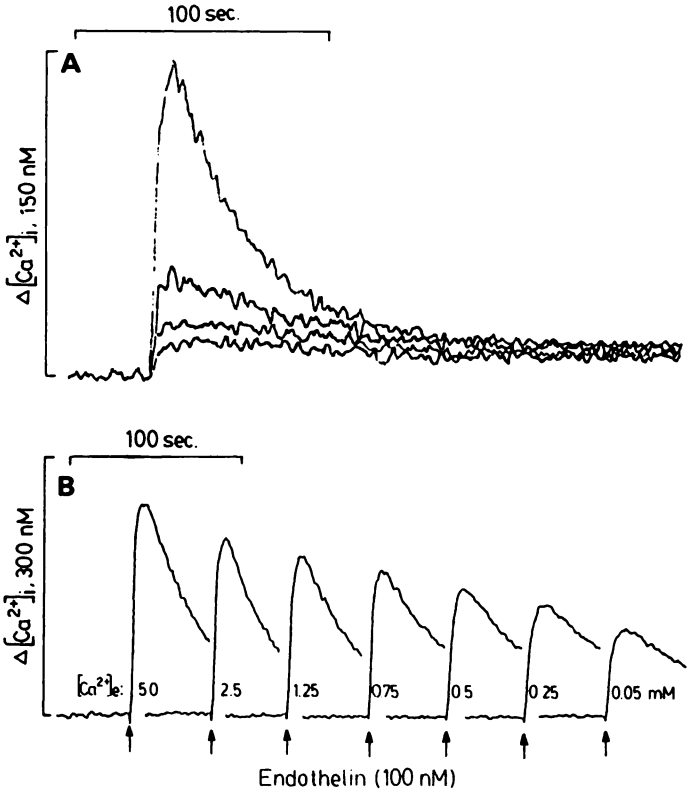

Fig. 1. Effects of vasoactive peptides on cytoplasmic $[Ca^{2+}]$ responses in pituitary cells. A, Comparison of the potencies of vasoactive peptides as stimuli of $[Ca^{2+}]_i$. Typical responses to ET-1 (100 nM), AII (1 μM), VP (1 μM), and noradrenalin (10 μM) are shown from *top* to *bottom*, respectively. B, Dose-dependent effects of extracellular Ca^{2+} on the amplitude of cytoplasmic Ca^{2+} responses in ET-stimulated pituitary cells. The suspension of pituitary cells from castrated animals was stimulated by ET in the indicated concentrations of extracellular calcium. $[Ca^{2+}]_i$ was assayed by fluorescence analysis, utilizing fura-2/acetoxy methyl ester (see Experimental Procedures for details). The *tracings* are representative of three different experiments.

to elicit a full $[Ca^{2+}]_i$ response is dependent on calcium entry as well as mobilization of intracellular calcium. As shown in Fig. 1B, a decrease in $[Ca^{2+}]_e$ (from 5.0 to 0.05 mM) was accompanied by a progressive decline in the peak amplitude of the ET-induced $[Ca^{2+}]_i$ response.

Three other vasoactive peptides, AII, VP, and noradrenalin, also induced increases in $[Ca^{2+}]_i$. However, their potencies and efficacies were much less than those of ET (Fig. 1A), indicating the difference in distribution of ET and other receptors for vasoconstrictor agonists within the subpopulations of pituitary cells. In support of this conclusion, ET-1 acts as a potent LH secretagogue in perifused cells, whereas two other vasoconstrictors, noradrenalin and VP, elicited only a small LH response and AII did not affect LH release (Table 1). The effect of VP on LH release was also evident in static cultures, whereas AII and noradrenalin did not activate LH exocytosis during 3-hr incubations (Table 1). These data suggest that ET is the major vasoactive hormone participating in the control of gonadotropin secretion.

ET- and GnRH-stimulated $[Ca^{2+}]_i$ responses in single gonadotrophs. In contrast to the increasing amplitude of $[Ca^{2+}]_i$ responses to increasing agonist concentrations in suspensions of enriched gonadotrophs (15), the $[Ca^{2+}]_i$ responses measured in single gonadotrophs are more complex and are analogous to action potentials. As shown in Fig. 2, A and B, small and transient increases in $[Ca^{2+}]_i$ were observed on stimulation with low (10 and 100 pM) doses of ET-1. However, 1 nM ET-1 induced an oscillatory pattern in $[Ca^{2+}]_i$, with maximal amplitude at the onset of stimulation, followed by progressive attenuation of the response during continuous stimulation. Further increases in ET-1 concentration were followed by increases in the frequency but not the amplitude of the Ca^{2+} oscillations (Fig. 2, C–F). The highest frequency that could be reached was about 20 spikes/min; an additional increase in ET-1 concentration was followed by "summation" of the spikes to give a biphasic response (Fig. 2G), similar to that observed in cell suspensions.

Such dose dependent actions of ET action on $[Ca^{2+}]_i$ indicates the existence of both subthreshold and threshold responses to ET. The subthreshold doses of ET do not trigger the oscillatory or biphasic responses but induce increases in $[Ca^{2+}]_i$ of much smaller amplitudes (Fig. 2, A and B). When the threshold is reached, Ca^{2+} spiking is initiated and the oscillatory response is observed. Individual cells varied in the threshold dose of agonist (between 10 and 1000 pM), the amplitude (between 350 and 1100 nM), and the frequency of their Ca^{2+} responses (between 2 and 25 min^{-1}). However, no effects of peptide concentration on the amplitude of the oscillatory responses were observed [net $[Ca^{2+}]_i$ amplitudes (nM): 798 ± 49 (n = 38) for 100 nM ET; 742 ± 83 (n = 16) for 10 nM ET; 806 ± 98 (n = 12) for 1 nM ET]. On the other hand, increased frequency of Ca^{2+} spiking was observed with increasing doses of ET [frequency (min^{-1}): 100 nM, 14 ± 1 (n = 32) or the biphasic response; 10 nM, 7 ± 1 (n = 16); 1 nM, 5 ± 1 (n = 15); 0.1 nM, 2 ± 0.4 (n = 5)].

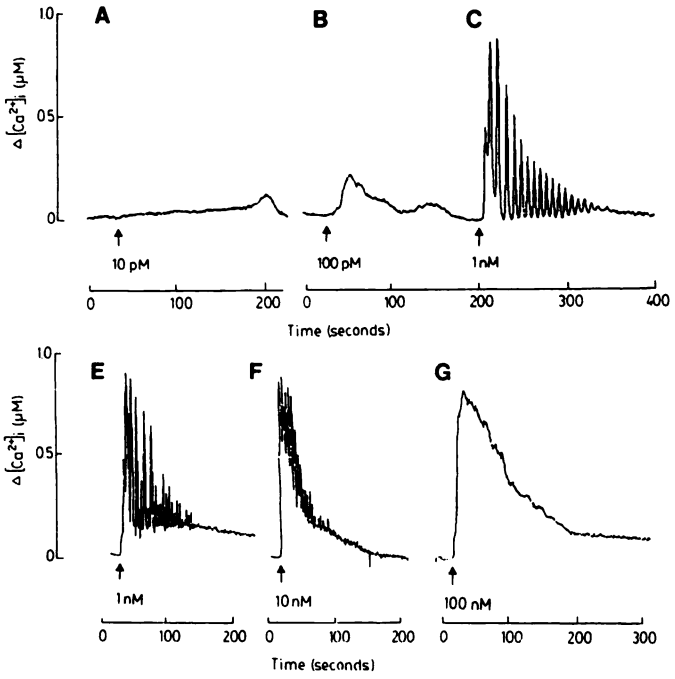

Fig. 2. Dose-dependent effects of ET on cytoplasmic $[Ca^{2+}]$ responses in single gonadotrophs. Local $[Ca^{2+}]_i$ responses were evoked by subthreshold doses of ET-1 (A, B) and threshold-oscillatory (C–F) and biphasic responses (G) by higher doses of ET-1. Experiments were performed in the presence of 1.2 mM extracellular Ca^{2+} at 24°. Oscillations in $[Ca^{2+}]_i$ or the spike phases were also observed under Ca^{2+}-deficient conditions, but without the plateau phase during prolonged stimulation (longer than 200 sec) with ET-1 (not shown). The data shown are typical of 50 records in gonadotrophs from different batches of cells from castrated females. Similar $[Ca^{2+}]_i$ responses to ET-1 were observed in gonadotrophs from normal females or males. Gonadotrophs were characterized by their $[Ca^{2+}]_i$ responses to transient exposure to 10 pM GnRH. $[Ca^{2+}]_i$ was calculated as described in Experimental Procedures.

The apparent discrepancy between $[Ca^{2+}]_i$ measurements in cell suspensions (amplitude modulated) and single cells (frequency modulated) could result from two factors, the variability in the lag time of the onset of response to low doses of ET-1 and the presence of different sub- and suprathreshold responses in single gonadotrophs. For example, the onset of the Ca^{2+} response was between 10 and 200 sec at 1 nM, between 5 and 60 sec at 10 nM, and between 1 and 10 sec at 100 nM ET-1. The responses observed in cell suspensions represent the means of the cells that are active at a given concentration, leading to the amplitude-modulated $[Ca^{2+}]_i$ responses observed in populations containing 2×10^6 cells (16).

Oscillations and the spike phase of the biphasic responses to ET were also seen under Ca^{2+}-deficient conditions (Fig. 3), indicating the major role of Ca^{2+} mobilization in agonist action. The ability of the gonadotrophs to maintain the same frequency of Ca^{2+} spikes during stimulation under extracellular Ca^{2+}-

TABLE 1

LH secretory responses of cultured pituitary cells during short and long term stimulation with neuropeptides and noradrenalin

Stimulus (100 nM)	Perfusion system			Static culture	
	LH release[a]				
	n	Peak	Plateau	n	LH release
		ng/ml			ng/ml/3 hr
GnRH	16	72.2 ± 6.5	28.0 ± 2.0	4	81.8 ± 7.5[b]
ET-1	6	54.5 ± 6.1	5.4 ± 1.1[c]	4	16.3 ± 0.7[b]
VP	5	6.9 ± 3.6	1.3 ± 0.5	4	7.2 ± 0.5[b]
Noradrenalin	4	6.2 ± 2.3	1.0 ± 0.9	4	5.4 ± 0.3
AII	3	1.6 ± 0.7	1.3 ± 1.3	4	5.8 ± 0.6
Basal	34	1.5 ± 0.8	1.4 ± 0.9	4	4.7 ± 0.4

[a] Values expressed as mean ± standard error above the basal release.
[b] $p < 0.01$ versus basal.
[c] $p < 0.01$ versus GnRH.

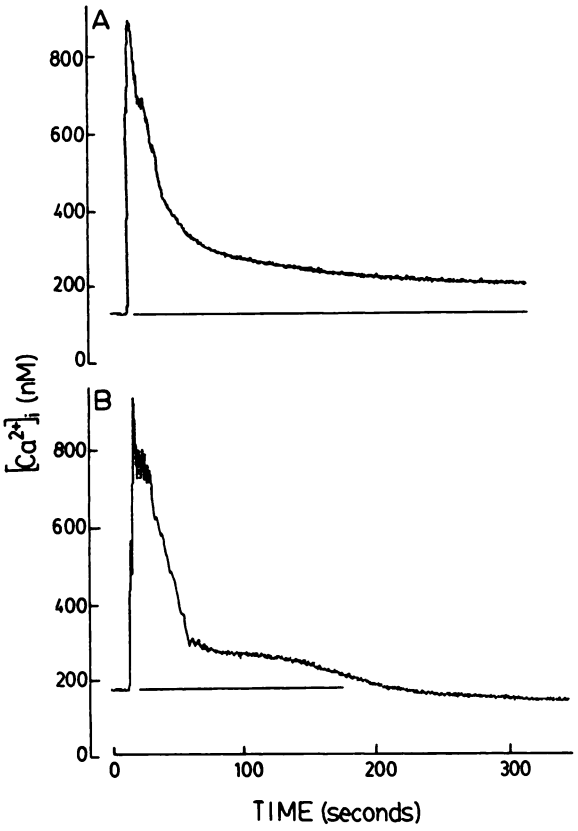

Fig. 3. Extracellular Ca^{2+} dependence of ET-1-induced cytosolic calcium responses. A, $[Ca^{2+}]_e$ = 1.25 mM; B, $[Ca^{2+}]_e$ = 200 nM.

deficient conditions [10 nM ET-1 in Ca^{2+}-deficient medium plus 100 μM EGTA, 7.1 ± 0.8 min^{-1} (n = 5), versus 10 nM ET-1 in 1.25 mM Ca^{2+}, 6.9 ± 0.7 min^{-1} (n = 16); not significant] suggests that calcium influx is not involved in the determination of the spike interval. However, the plateau phase of Ca^{2+} responses is abolished under extracellular Ca^{2+}-deficient conditions (Fig. 3), supporting the view that Ca^{2+} entry is responsible for the maintenance of Ca^{2+} signal in ET- and GnRH-stimulated cells.

Such findings contrast with observations in other cell types, in which the frequency or the amplitude of Ca^{2+} spikes was affected when the cells were stimulated in Ca^{2+}-deficient medium. In hepatocytes, no decrease in the amplitude of Ca^{2+} transients was observed during prolonged stimulation (17), consistent with equilibrium between efflux and influx of Ca^{2+} in those cells. In agreement with this, synchronized oscillations of Ca^{2+} entry and Ca^{2+} release have been reported to be present in agonist-stimulated pancreatic acinar cells (18). On the other hand, the progressive decrease of the spikes in ET-stimulated gonadotrophs, independent of $[Ca^{2+}]_e$, indicates that efflux predominates over influx during agonist stimulation, leading to emptying of the releasable Ca^{2+} pool.

To examine the status of the residual agonist-sensitive Ca^{2+} pool, we restimulated gonadotrophs during the plateau phase of their Ca^{2+} responses to the two agonists. A high concentration (100 nM) of ET-1 elicited further increases in $[Ca^{2+}]_i$ in single gonadotrophs that were prestimulated with low doses of the peptide, but such responses were much smaller than those initially elicited by ET [Fig. 4, A, second stimulus, versus C, first stimulus; net amplitudes (nM): 798 ± 49 (n = 38) versus 349 ± 29 (n = 5), respectively; $p < 0.001$]. Although very small, Ca^{2+} responses to high GnRH concentrations in cells pretreated with low GnRH doses were also observed (Fig. 4, B, second stimulus, versus D, first stimulus). In contrast, further increases in agonist concentrations elicited little or no response in gonadotrophs that were preexposed to high doses of ET or GnRH (Fig. 4, C and D).

In general, the diminished ability of each agonist to restimulate Ca^{2+} release could be related not only to the emptying of the agonist-sensitive Ca^{2+} pool during the previous stimulus but also to the rapid desensitization by the agonist. The fact that both peptides (GnRH and ET) operate through the same second messenger system in the same cell type was useful for addressing the extent to which desensitization participates in these effects. In experiments with combinations of agonists, GnRH increased $[Ca^{2+}]_i$ in cells that were pretreated with low (Fig. 5A, *trace a*) or high ET doses (Fig. 5A, *traces b, c,* and *d*), but ET-1 produced only small or no increases in $[Ca^{2+}]_i$ in cells that were prestimulated with low or high doses of GnRH (Fig. 5B, *trace a*, versus *traces b, c,* and *d*).

The Ca^{2+} ionophore ionomycin is a potent stimulator of Ca^{2+} release from intracellular stores, as well as Ca^{2+} entry from the extracellular medium. In single pituitary gonadotrophs, 5 μM ionomycin produced a transient biphasic response, with a Ca^{2+} spike followed by a low sustained phase (Fig. 6, A and B). In the absence of extracellular Ca^{2+} ($[Ca^{2+}]_e$ = 200 nM), the amplitude of the Ca^{2+} response to ionomycin was 70–75% (Fig. 6B) of that seen in the presence of extracellular Ca^{2+} (Fig. 6A), whereas the profiles of both responses were similar. The transient response to ionomycin was observed over the dose range of 0.5 to 15 μM, with proportional increases in the amplitudes of responses. In extracellular Ca^{2+}-deficient medium, further increases in ionomycin concentration were followed by continuous elevation of $[Ca^{2+}]_i$ during the 400 sec of stimulation, implying that release of Ca^{2+} occurs not only from endoplasmic reticulum but also from other cellular stores.

The averaged amplitude of ionomycin (5 μM)-induced increases in $[Ca^{2+}]_i$ in the absence of extracellular calcium was 590 + 31 nM (n = 5). Like GnRH, ionomycin was able to induce further mobilization of Ca^{2+} in ET-1 (10 nM)-stimulated cells, but of much smaller amplitude than that in controls [350 ± 56 nM (n = 3); $p < 0.001$; Fig. 6C]. After high doses of ET (100 nM), ionomycin (Fig. 6E), like GnRH (Fig. 5A), could still induce release of Ca^{2+}, but of much smaller amplitude [145 ± 33 nM (n = 3)]. In GnRH-prestimulated cells, however, ionomycin induced further release of Ca^{2+} only if the frequency of the agonist-induced Ca^{2+} oscillations was low and the duration of the signal was short. Otherwise, the drug had little or no effect on the $[Ca^{2+}]_i$ profile induced by GnRH (Fig. 6, D and F).

These data suggest that the inability of GnRH, ET, or ionomycin to reinitiate Ca^{2+} responses in GnRH-stimulated cells is due to complete discharge of the agonist-releasable Ca^{2+} pool in such cells. In another words, Ca^{2+} efflux predominates over influx during initiation of $[Ca^{2+}]_i$ responses, and the plateau phase represents the equilibrium between Ca^{2+} efflux and influx that is reached during sustained agonist stimulation. A similar phenomenon occurs in ET-stimulated cells, in agreement with the view that initiation of Ca^{2+} responses by the two peptides does not differ. However, the ability of GnRH and ionomycin to reinitiate Ca^{2+} release in high dose ET-stimulated

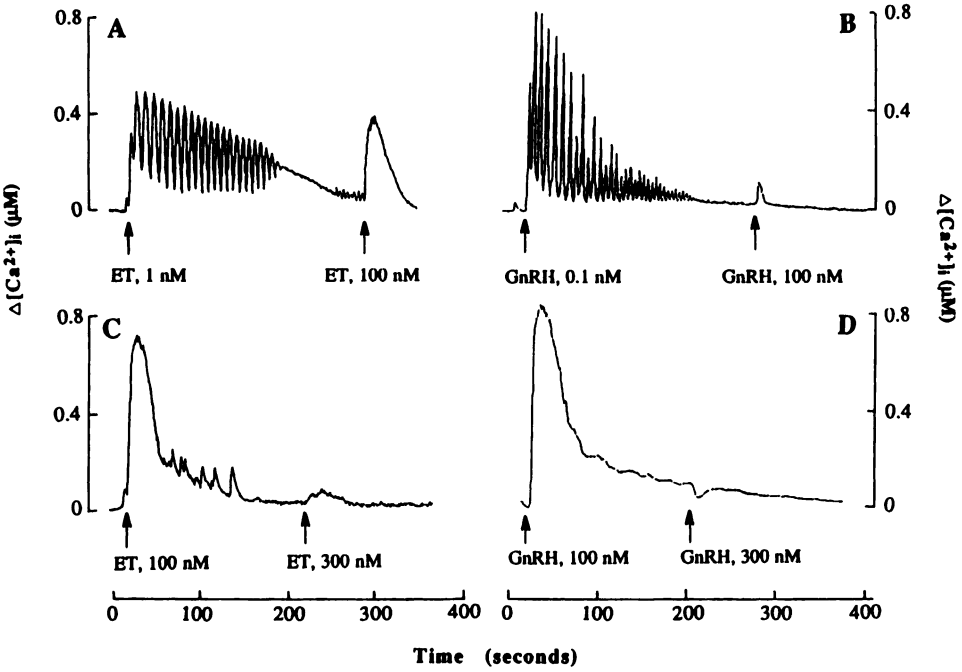

Fig. 4. Effects of continuous stimulation of pituitary cells with GnRH or ET on subsequent $[Ca^{2+}]_i$ responses to the same peptide. ET-1 (A) or GnRH (B) was added in 0.1 nM or 100 nM doses at the beginning of the experiments and again at 200 sec, in the presence of 1.2 mM Ca^{2+}. The *tracings* represent single-cell recordings in identified gonadotrophs (see the legend to Fig. 2).

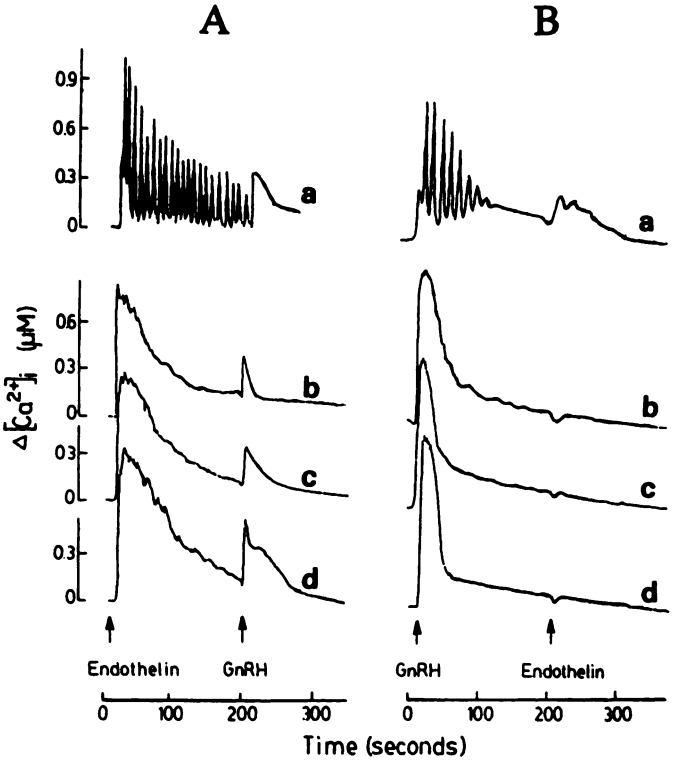

Fig. 5. Effects of stimulation of gonadotrophs with GnRH or ET-1 on subsequent $[Ca^{2+}]_i$ responses to the other peptide (cross-reactivation). ET-1 (A) was added in 0.1 nM (*trace a*) or 100 nM (*traces b, c,* and *d*) doses at the beginning of the experiments, and GnRH (100 nM in all cases) was added 200 sec later. In the converse experiments, GnRH (B) was added at 0.1 nM (*trace a*) or 100 nM (*traces b, c,* and *d*) at the beginning, and ET (100 nM) was added 200 sec later.

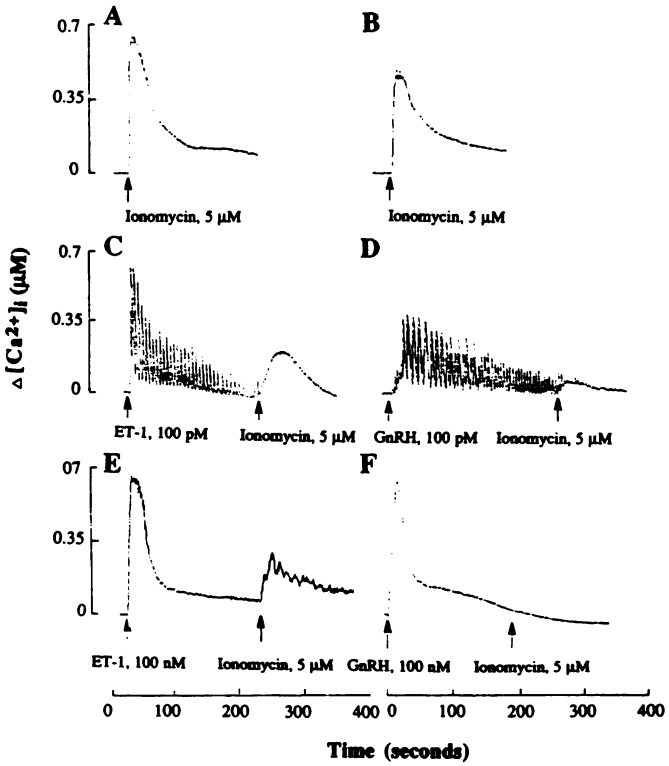

Fig. 6. Effects of ionomycin on cytosolic calcium in single gonadotrophs, unstimulated or continuously stimulated with GnRH or ET. Ionomycin (5 μM) was added in the presence of 1.2 mM Ca^{2+} (A) or in calcium-deficient medium (100 nM) (B). Stimulation by ET (C and E) or GnRH (D and F) was performed under Ca^{2+}-deficient conditions. Two hundred seconds after stimulation with neuropeptides, the cells were exposed to 5 μM ionomycin. The data shown are representative of three experiments.

cells demonstrates that the agonist-sensitive pool is still partially available in such cells and suggests that receptors for the two peptides undergo different mechanism and/or kinetics of desensitization during continuous stimulation.

In agreement with this proposal, gonadotrophs continuously exposed to GnRH or ET-1 for 30 min, followed by a 30-min recovery period, respond differently to subsequent stimulation with the same agonist. Whereas GnRH was able to induce normal biphasic Ca^{2+} responses in such cells (Fig. 7, A versus

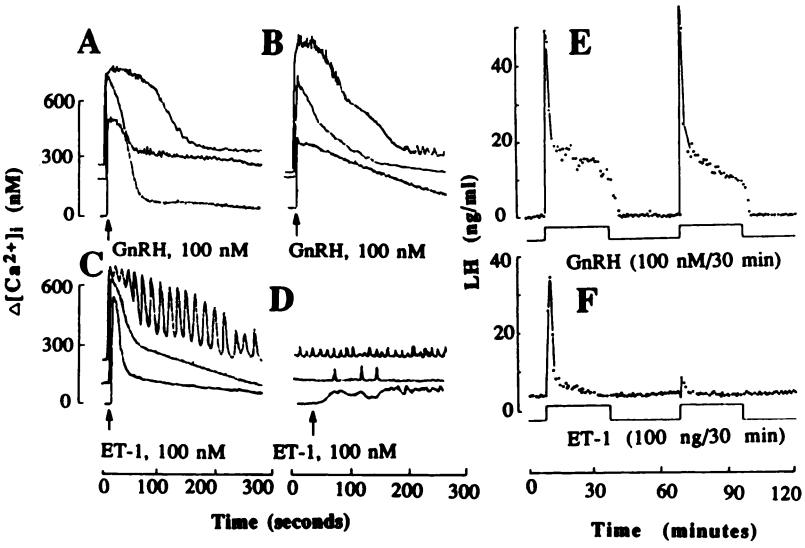

Fig. 7. Cytoplasmic $[Ca^{2+}]_i$ and gonadotropin responses to repetitive stimulation of perifused pituitary cells with GnRH and ET. Cells were stimulated with GnRH (A) or ET-1 (C) at 100 nM for 30 min. $[Ca^{2+}]_i$ response was followed only during the first 300 sec. After 30 min of stimulation, the cells were washed and restimulated with the same concentration of agonists (B and D). The *tracings* shown represent recordings from six single gonadotrophs and are typical of 30 records. For secretory studies, cells were perifused with warm (37°) medium at a flow rate of 0.6 ml/min for 1 hr before stimulation, to establish a stable baseline. Fractions were collected every minute during the test period. The data shown are representative of four experiments.

B), additional stimulation with ET was ineffective or elicited subthreshold $[Ca^{2+}]_i$ responses (Fig. 7, C versus D). In contrast, ionomycin (5 μM) was able to elicit Ca^{2+} release of the expected amplitude in cells that were preexposed for 30 min to either 100 nM ET GnRH, followed by a 30-min recovery period [net amplitude (nM): controls, 590 ± 31 (n = 5); ET-treated, 510 ± 42 nM (n = 3); GnRH-treated, 525 ± 36 nM (n = 3)]. These data indicate that refilling of the agonist-sensitive Ca^{2+} pool occurs relatively rapidly in both GnRH- and ET stimulated cells. However, the ET receptor pathway in gonadotrophs undergoes rapid desensitization that is responsible for the observed effects on Ca^{2+} signaling.

The LH secretory responses to the two agonist peptides also differed in certain respects. During short term stimulation (10 min) of perfused pituitary cells, the peak LH values did not show a significant difference, but the plateau phase of ET-1-induced LH release was always smaller than that elicited by GnRH (Table 1; Fig. 7, E and F). The LH response to ET-1 decreased progressively during prolonged stimulation, reaching the basal level after 15–20 min of exposure to the peptide (Fig. 7F). The ability of ET-1 to induce LH release during a second period of stimulation (30 min after washing) was almost abolished (Fig. 7F), whereas the response to GnRH, albeit reduced, was still substantial (Fig. 7E). During long term stimulation (3 hr) in static cultures, the response to ET-1 was 15–20% of that elicited by GnRH (Table 1), again indicating that occupancy of specific calcium-mobilizing receptors by the two peptide agonists has similar short term effects, but with different mechanisms and/or kinetics of desensitization during prolonged stimulation.

Endocytosis of ^{125}I-ET-1 receptors. Measurements of ^{125}I-ET-1 binding and internalization suggest that the rapid endocytosis of ET receptors contributes to the desensitization of the ET response. As shown in Fig. 8, the amount of cell-bound radioactivity could be separated into acid-sensitive and acid-resistant components, the former corresponding to membrane-bound ET and the latter indicative of internalized receptors. The sum of these two components is quantitatively equivalent to the total cellular binding, estimated in cells lysed without prior acid treatment (Fig. 8, *right*). At 37° about 50% of the bound radioactivity was acid resistant, whereas at 4° 5% of the ^{125}I-ET-1 binding was acid resistant (not shown). However, the total binding at 4° was only 20% of that observed at 37° after 60 min of incubation time (data not shown). In another series of experiments, ligand compartmentalization was estimated by brief trypsinization and revealed similar proportions of cell surface binding and internalization of bound ^{125}I-ET-1 as those observed with acid treatment (not shown).

ET-induced secretory responses in individual pituitary cell types. The prominent ET-1-induced $[Ca^{2+}]_i$ responses in pituitary cell suspensions, in comparison with those elicited by other vasoactive peptides as well as GnRH (Fig. 1A), suggested that ET-1 acts not only on gonadotrophs but also on other subpopulations of pituitary cells. We utilized the fact that ET-1 was unable to induce further increases in $[Ca^{2+}]_i$ in GnRH-stimulated gonadotrophs (Figs. 4–6) to examine its possible actions on other pituitary cells. As shown in Fig. 9, in a pituitary cell suspension previously stimulated with a high (100 nM) concentration of GnRH, ET was still able to induce a substantial $[Ca^{2+}]_i$ response. To evaluate the secretory actions of ET on other hormone-producing cells, we analyzed the LH, FSH, TSH, GH, PRL, and ACTH responses of perifused pituitary cells to 100 nM ET-1. As shown in Fig. 10, all of the secretory cell types exhibited rapid but short-lived responses to ET, with marked differences in the amplitudes of the individual responses (expressed as ratios over basal secretion).

Discussion

Specific binding sites for ET-1 are widely distributed in tissues, including heart, lung, kidney, and the central nervous system (19). ET-1 binding sites are also present in the pituitary gland (11, 15) and are coupled to the phospholipase C-mediated hydrolysis of polyphosphoinositides, with production of the two second messengers Ins(1,4,5)P_3 and diacylglycerol (15). The present data have shown that pituitary cells respond by increases in $[Ca^{2+}]_i$ and exocytosis during stimulation with ET-1; pituitary cells secreting glycoprotein hormones (LH, FSH, and TSH) are the most sensitive to ET, whereas corticotrophs are almost unresponsive to ET. Three other vasoactive peptides, AII, VP, and noradrenalin, also induced rises in $[Ca^{2+}]_i$, but of much smaller amplitude. Our data show that AII and

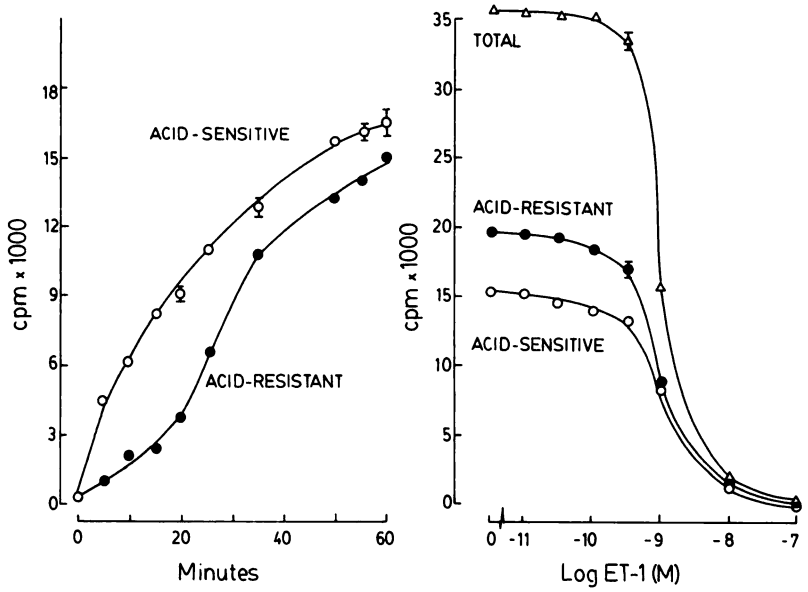

Fig. 8. Kinetics of binding and internalization of ^{125}I-ET-1 in pituitary cells. A, At the indicated time, the unbound hormone was removed and ET-1 bound to the cell surface (acid-sensitive) and cell-associated radioactivity (acid-resistant) were estimated as described in Experimental Procedures. B, The cell monolayers were incubated at 37° for 60 min in the presence of unlabeled ET-1 at the doses indicated. Total cell-bound radioactivity was estimated in solubilized cells without prior acid treatment. Data shown (mean ± standard error, three determinations) are representative of three similar experiments.

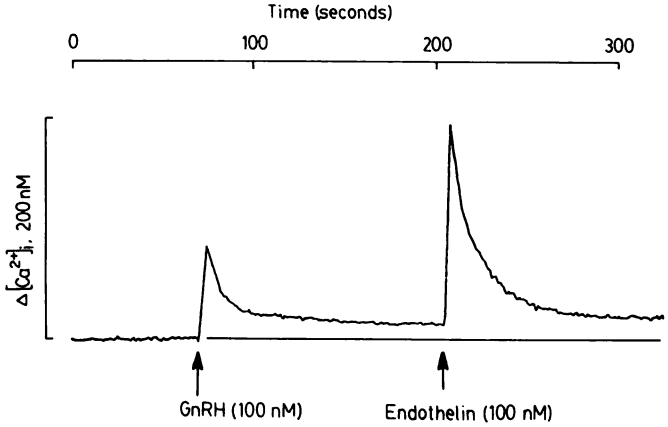

Fig. 9. $[Ca^{2+}]_i$ responses to GnRH and ET of pituitary cells in suspension. The data shown are representative of experiments performed with similar results in three different batches of pituitary cells.

noradrenalin have practically no effects on LH and FSH release, whereas VP has only a small effect at high concentrations, in agreement with previously published observations (20). In contrast, corticotrophs are sensitive to VP (21), and AII is a potent stimulus of PRL secretion (22). Such data indicate the differential distribution of receptors for vasoactive peptides among the individual pituitary cell types.

The most potent LH secretagogue among the vasoconstrictor agonists, ET, induces a complex pattern of Ca^{2+} responses in single gonadotrophs and pituitary cell suspensions, according to dose, temperature, and $[Ca^{2+}]_e$. Of particular interest were the several $[Ca^{2+}]_i$ profiles observed on analysis of Ca^{2+} responses in single gonadotrophs. In general, the $[Ca^{2+}]_i$ responses to increasing doses of ET were of three main types, as follows: subthreshold responses, with increased amplitude and no Ca^{2+} oscillations; a threshold-oscillatory pattern, with modulation of frequency but not amplitude by increasing agonist concentrations; and threshold-biphasic responses that resemble the $[Ca^{2+}]_i$ [and $Ins(1,4,5)P_3$] responses seen in cell suspensions. The similarities between these responses and those seen during action potentials are striking. However, all such responses were also seen under extracellular Ca^{2+}-deficient conditions, indicating that the underlying mechanism is inositol trisphosphate-induced Ca^{2+} mobilization. In contrast, ET-induced oscillations in $[Ca^{2+}]_i$ in A10 vascular smooth muscle cells (23), like those

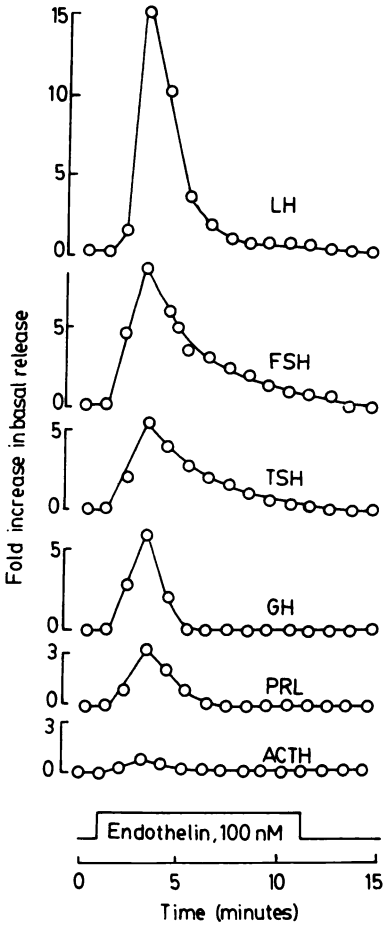

Fig. 10. Stimulation of pituitary hormone release by ET. LH, FSH, TSH, GH, PRL, and ACTH levels were measured in the effluent during column perifusion of 15 × 10^6 cells attached to Cytodex beads. Fractions were collected at 1-min intervals. Data points are the means of duplicate incubations in one of three similar experiments.

seen in untreated (24) and agonist-stimulated somatotrophs (25), are predominantly extracellular Ca^{2+} dependent.

The maneuvers with $[Ca^{2+}]_e$ and restimulation of the cells with GnRH, high ET, and ionomycin suggested that ET-induced mobilization of Ca^{2+} from intracellular stores occurs only during the first 200–400 sec of agonist stimulation, depending on the dose of the peptides. On the other hand, an ET-activated Ca^{2+} entry pathway is responsible for the plateau phase of the biphasic responses at high agonist concentrations and for the plateau phase that occurs after the oscillatory response at lower agonist doses. However, such entry does not prevent the progressive decrease in amplitude of the frequent Ca^{2+} spikes during agonist stimulation. This fact, as well as the absence of a rise in $[Ca^{2+}]_i$ during subsequent exposure to GnRH and ET, or to ionomycin under extracellular Ca^{2+}-deficient conditions, indicates that the capacity of the agonist-sensitive Ca^{2+} pool is limited, and that the equilibrium between Ca^{2+} entry and the activity of the plasma membrane Ca^{2+} pump is just sufficient to maintain the low plateau phase of the $[Ca^{2+}]_i$ response. Otherwise, the agonist-induced oscillations in $[Ca^{2+}]_i$ would be more prolonged, and agents like GnRH or ionomycin would elicit a second rise in $[Ca^{2+}]_i$.

The decrease in the amplitudes of Ca^{2+} spikes during prolonged stimulation with ET contrasts with observations in other cell types, in which Ca^{2+} oscillations occur with no decrease in amplitude if extracellular Ca^{2+} is present. The discrepancy between the oscillatory pattern in gonadotrophs and such cells, for example hepatocytes (17), could be related to the frequency of Ca^{2+} spikes and the capacity of Ca^{2+} entry pathway(s). In hepatocytes, the frequency of Ca^{2+} spiking is very low (~2/min), and Ca^{2+} may refill the agonist-sensitive pool between oscillations. In agreement with this, synchronized oscillations of both Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} entry have been recently observed in pancreatic acinar cells (18). In contrast, the frequency of the oscillations elicited by agonists in gonadotrophs is much higher than that seen in hepatocytes, reaching 20–25 transients/min, and the capacity of Ca^{2+} entry pathway(s) is not sufficient to sustain such high frequencies of calcium spiking.

The initiation of Ca^{2+} responses in ET-stimulated single cells is similar to that seen in GnRH-stimulated gonadotrophs. However, there are significant differences during responses to prolonged and repetitive stimulation with the two agonists. For example, GnRH is able to reactivate the Ca^{2+} mobilization pathway in cells stimulated with low or high doses of ET (with the expected decrease in amplitude of the response), but not *vice versa*. Furthermore, re-exposure of washed cells to the same peptide 30 min after prior stimulation clearly shows significant recovery of the ability of GnRH, but, again, not of ET, to mobilize Ca^{2+}. Such observations indicate that different and/or additional mechanisms, or differences in the kinetics of these mechanisms, participate in the marked attenuation of ET action, compared with that of GnRH.

The physiological consequences of these differences are evident in the secretory responses to short and long term stimulation by the two hormones. Both neuropeptides exhibit similar secretory activities in cell perifusion studies during short term stimulation. No significant differences in the spike phase of secretion, which correlates with the profiles of the Ca^{2+} responses, were seen in GnRH- or ET-stimulated cells. However, the plateau phase of ET-induced LH release was significantly smaller and returned to the basal level after 10–15 min during prolonged stimulation. In addition, repetitive stimulation with ET was accompanied by more rapid and profound desensitization of the secretory response. Under static culture conditions, GnRH-induced LH release is maintained in a dose-dependent manner over several hours in cultured pituitary cells (16), whereas the stimulatory action of ET is of limited duration. The rapid development of desensitization to high concentrations of ET, in addition to the possible degradation of the peptide, could account for the lack of effect of ET on LH, TSH, and PRL release during 4-hr incubations with pituitary tissue (11) and for the relatively smaller LH responses that we (15) and others (13) have observed during 3-hr stimulation of pituitary cell cultures. In agreement with these observations, measurements of ET-1 binding and internalization have shown the development of rapid (within 30 min) endocytosis of ET receptors. However, other steps in receptor coupling to LH exocytosis must also be involved in the desensitization process, and their clarification requires more detailed analysis.

In summary, these observations have demonstrated that in pituitary cells, as in vascular smooth muscle, ET shares with other vasoactive hormones, such as AII, VP, and noradrenalin, the ability to increase $[Ca^{2+}]_i$. This initial signaling event leads to short term stimulation of pituitary hormone secretion, especially in gonadotrophs. The differences in the secretory potency of ET on the various pituitary cell types, as compared with other constrictor agonists, reflects the distribution of ET receptors within the subpopulations of pituitary cells, as well as their cellular activation mechanisms. In gonadotrophs, ET is the most potent of the vasoactive peptides that elicit gonadotropin secretion; VP and noradrenalin have small effects at high concentrations, and AII is almost inactive. Although ET and GnRH share a common signal transduction pathway, rapid and marked desensitization of the ET-induced $[Ca^{2+}]_i$ response is responsible for the reduced efficacy of the vasoconstrictor peptide during sustained stimulation of gonadotropin secretion.

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