Relaxin Secretion by Porcine Large Luteal Cells: Effect of Protein Synthesis Inhibitors

(43520)

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Abstract. **The purpose of the experiments reported herein was to investigate the relative importance of new hormone synthesis to basal and prostaglandin E_2-stimulated rates of relaxin release. A relaxin-reverse hemolytic plaque assay was used to monitor relaxin release from individual large luteal cells (LLC) in which new protein synthesis was inhibited by cycloheximide or actinomycin D. These treatments significantly decreased the rate of relaxin release. In addition, cycloheximide reduced the total fraction of LLC possessing the ability to form plaques by about 10%, suggesting complete suppression of relaxin from this subset of cells. Exposure of inhibitor-treated LLC to prostaglandin E_2 (a relaxin stimulatory secretagogue) enhanced relaxin release, and restored suppressed LLC back into the secretory population. Taken overall, these results demonstrate that the majority of relaxin-releasing LLC exploit a mixture of newly synthesized and older, stored hormone to achieve basal secretion. A minority of relaxin-releasing LLC, however, appear to depend wholly on newly synthesized hormone for basal secretion. The differential activity (and interaction) of these pathways in individual LLC may provide a potential explanation for the markedly heterogenous manner of hormone release observed in this (and other) cell types, and for the action of relaxin secretagogues.** [P.S.E.B.M. 1993, Vol 202]

Considerable evidence supports the contention that endocrine cells of pituitary and pancreatic origin preferentially release newly synthesized protein hormone under basal conditions (1, 2). More recent data suggest that individual cells differ in the nature of released hormone; some pituitary cells were reported to utilize exclusively newly synthesized hormone for basal release, whereas other cells released both new and older, stored hormone under the same conditions (3).

Porcine large luteal cells (LLC) synthesize, store, and release considerable amounts of the insulin-like hormone relaxin (4–6). They share with pituitary cells the secretory characteristic of functional heterogeneity, i.e., individual LLC release strikingly different amounts of relaxin under basal and modulated conditions (7, 8). But in contrast to pituitary cells, there is little information concerning the biosynthesis and trafficking of this ovarian hormone. In particular, the relative importance of new hormone synthesis and stored hormone to relaxin release has not been investigated at the level of the single cell. To investigate this problem, we adopted essentially the same approach successfully exploited by Chen *et al.* (3) to address the same questions in a pituitary cell system, namely the use of a reverse hemolytic plaque assay in association with inhibitors of protein synthesis. This strategy permitted the detection of relaxin release, at the single cell level, from LLC in which protein synthesis was inhibited.

Material and Methods

Release of relaxin by individual LLC in monolayer culture was detected through use of a relaxin-reverse hemolytic plaque assay. Ovaries were obtained from pregnant pigs (Day 30–40 of gestation). Full details of the methods of tissue collection, dispersion into single cell suspension, and execution of the relaxin-reverse hemolytic plaque assay are provided elsewhere (7, 8).

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Received February 5, 1992. [P.S.E.B.M. 1993, Vol 202]
Accepted July 9, 1992.

0037-9727/93/2022-0148$3.00/0

All experiments were carried out on three to four separate occasions, using cells derived from entirely different donors. Results are expressed as mean ± SE. Statistical differences between control and treatment groups were determined by analysis of variance, followed by the Newman-Keul range test. A separate analysis of variance was conducted at each sampling time during the experimental incubation.

Results

Effect of Graded Concentrations of Cycloheximide on Basal Relaxin Release. In the presence of cycloheximide, a dose-related inhibition in the rate of plaque formation under basal conditions was observed (Table I). Concentrations of 0.01 μM and 0.1 μM cycloheximide did not significantly change the rate of plaque formation, as compared with controls. However, a concentration of 1 μM cycloheximide significantly reduced ($P < 0.05$) the number of plaque-forming LLC at 2 and 3 hr of incubation, but not at other time points. Concentrations of 10 μM cycloheximide significantly reduced ($P < 0.05$) the number of plaque-forming LLC at all time points of the incubation in the experiment illustrated, although the inhibitory effect at 1 hr of incubation was inconsistently present (compare Table I with Fig. 1). An increased concentration of cycloheximide (100 μM) did not significantly enhance this inhibitory effect. On the basis of these preliminary data, a concentration of 10 μM cycloheximide was identified as an approximate maximally effective dose with respect to relaxin release.

Effect of Cycloheximide on Basal and Stimulated Relaxin Release. Subsequent experiments were carried out to ascertain the relaxin secretory response to 10 μM cycloheximide under basal and prostaglandin (PG) E_2-stimulated conditions. In these investigations, a maximum of about 60% of all LLC possessed the capacity to form a plaque, and this fraction is consistent with previous studies (7). Maximal plaque formation occurred at 8 hr under control conditions since there was no significant increase in the fraction of LLC that formed plaques after this time (Fig. 1). Under basal (nonstimulated) conditions, an inhibitory effect of 10 μM cycloheximide on plaque formation was not detectable at the first time point (1 hr of incubation). Thereafter, however, (i.e., at 2–12 hr of incubation), the proportion of plaque-forming LLC present in cycloheximide-treated monolayers was significantly less than control values ($P < 0.05$). There were, for example, 40% fewer plaque-forming LLC in cycloheximide-treated monolayers at 4 hr of incubation, as compared with control values ($P < 0.05$). In addition, the sustained reduction in the percentage of plaque-forming LLC at the time point of maximal plaque formation, 8 hr (and also at the succeeding time point, 12 hr), demonstrates the presence of a subset of LLC (about 8% of all LLC) in which relaxin release was entirely suppressed by cycloheximide.

Treatment with the relaxin stimulatory secretagogue PGE_2 (0.1 μM) alone resulted in the anticipated increase in the rate of plaque formation. The percentage of plaque-forming LLC in monolayers bathed in PGE_2 alone was significantly higher ($P < 0.05$) than controls at 1–4 hr of incubation (Fig. 1); thus, maximal plaque formation was obtained at 4 hr (as compared with 8 hr for controls). There was no significant increase in plaque formation between 4, 8, and 12 hr of incubation in PGE_2-treated monolayers (Fig. 1), confirmation that PGE_2 treatment does not recruit additional LLC into the relaxin-releasing pool of LLC (8). Combinatorial treatment of monolayers with PGE_2 and cycloheximide resulted in a pattern of plaque formation that was not significantly different from that observed in monolayers treated with PGE_2 alone (Fig. 1).

Effect of Actinomycin D on Basal and PGE_2-Stimulated Relaxin Release. The experiments described above were repeated with actinomycin D to determine whether this agent (which blocks transcrip-

Table I. Effect of Graded Concentrations of Cycloheximide on the Rate of Plaque Formation in the Relaxin-Reverse Hemolytic Plaque Assay[a]

Treatment	Incubation time (hr)				
	1	2	3	4	8
Control	9.1 ± 0.6*	13.8 ± 1.1*	20.2 ± 1.1*	30.0 ± 1.2*	62.5 ± 1.7*
Cycloheximide					
0.01 μM	8.3 ± 0.7*	11.3 ± 0.8*†	19.9 ± 0.1*	28.0 ± 0.7*	59.4 ± 0.3*
0.1 μM	8.7 ± 1.2*	11.5 ± 0.4*	16.9 ± 1.3*,†	27.8 ± 1.8*	60.8 ± 0.4*
1 μM	7.8 ± 1.0*	9.0 ± 0.6†	16.3 ± 0.5†	27.7 ± 1.3*	59.7 ± 0.7*
10 μM	5.6 ± 0.9†	7.8 ± 0.5†,‡	11.7 ± 1.0‡	19.1 ± 0.8†	46.7 ± 0.9†
100 μM	3.3 ± 1.6†	6.6 ± 0.6‡	10.2 ± 0.9‡	22.1 ± 1.2†	48.7 ± 0.9†

[a] The data shown are the percentage of plaque-forming LLC (mean ± SE) in control and treated monolayers at various time points of the experimental incubation. Results are from a single experiment representative of two experiments. The cells were derived from a Day 36 pregnant pig, and triplicate monolayers were analyzed at each time point. Values denoted by a different symbol (*, †, ‡) are significantly different ($P < 0.05$) within the same column.

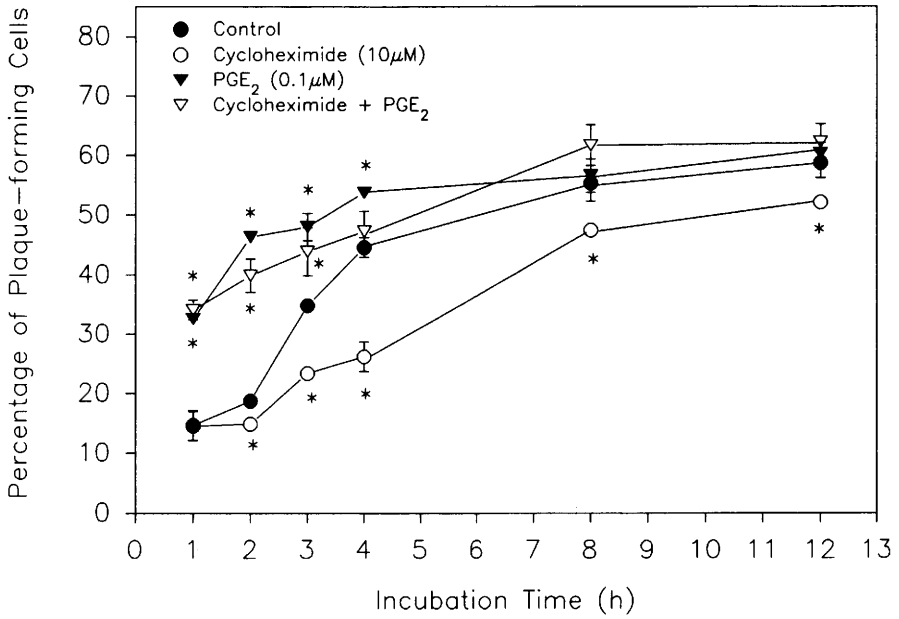

Figure 1. Effect of cycloheximide, PGE_2, or cycloheximide and PGE_2 combined on the rate of plaque formation. The data shown are the mean ± SE from three independent experiments, using cells obtained from Day 32–37 pregnant pigs. Where no error bar is shown, the SE is less than the size of the symbol. Values denoted by an asterisk are significantly different than control values ($P < 0.05$). There was no significant difference between the percentage of plaque-forming LLC in monolayers exposed to PGE_2 alone or to cycloheximide + PGE_2 (inverted triangles) at any time point.

tion) exerted the same effects as cycloheximide (which inhibits translation rather than the formation of new mRNA). Preliminary titration studies showed that 1 μM actinomycin D induced maximal effects on the rate of plaque formation. In these experiments, a maximum of nearly 60% of all LLC possessed the capacity to form a plaque under control conditions; this occurred at 12 hr of incubation, as indicated by the response to PGE_2 (Fig. 2). Under basal conditions, no effect of actinomycin D on plaque formation was detectable at the first time point (1 hr of incubation). However, treatment with 1 μM actinomycin D alone resulted in significant reduction in the fraction of plaque-forming LLC inhibition at 2–12 hr of incubation. There were, on average, 43% fewer plaque-forming LLC in actinomycin-treated monolayers at 4 hr of incubation, as compared with control values ($P < 0.05$). Treatment with PGE_2 (0.1 μM) resulted in an increase (as compared with controls) in the percentage of plaque-forming LLC at 2–8 hr of incubation; the failure to detect a response to PGE_2 at the first time point (compared with the experiments illustrated in Fig. 1) is not unusual and relates to animal-to-animal variation in release rates. Note that there was no significant increase in the percentage of plaque-forming LLC in PGE_2-treated monolayers between 8 and 12 hr of incubation, and no significant difference between the percentage of plaque-forming LLC in PGE_2-treated and control monolayers at 12 hr of incubation. Simultaneous treatment of monolayers with PGE_2/actinomycin D resulted in a pattern of plaque formation not significantly different from that of monolayers treated with PGE_2 alone (Fig. 2).

Discussion

In these studies, relaxin release from individual LLC treated with an inhibitor of protein synthesis, cycloheximide or actinomycin D, was monitored using a relaxin-reverse hemolytic plaque assay. Reverse hemolytic plaque assays provide the unique ability to monitor both basal and secretagogue-induced hormone release by individual cells in which protein synthesis is suppressed. This experimental paradigm permitted us, therefore, to track on a cell by cell basis the requirement for newly synthesized hormone. The results showed that the effect of two different inhibitors of protein synthesis was to reduce the percentage of plaque-forming cells over the course of the experimental incubation (as compared with control monolayers). This is unambiguous evidence of an inhibition of the rate of relaxin release by inhibitor-treated, relaxin-releasing LLC. In addition, relaxin release from a subset of LLC (about 10%) exposed to cycloheximide ceased entirely, since the percentage of plaque-forming LLC in inhibitor-treated monolayers was significantly less than control values at the point of maximal plaque formation. A relaxin secretagogue (PGE_2) enhanced the rate of relaxin release from cycloheximide- and actinomycin D-treated LLC. In addition, PGE_2 restored the fraction of relaxin-releasing LLC in cycloheximide-treated populations to normal.

The simplest interpretation of this information is

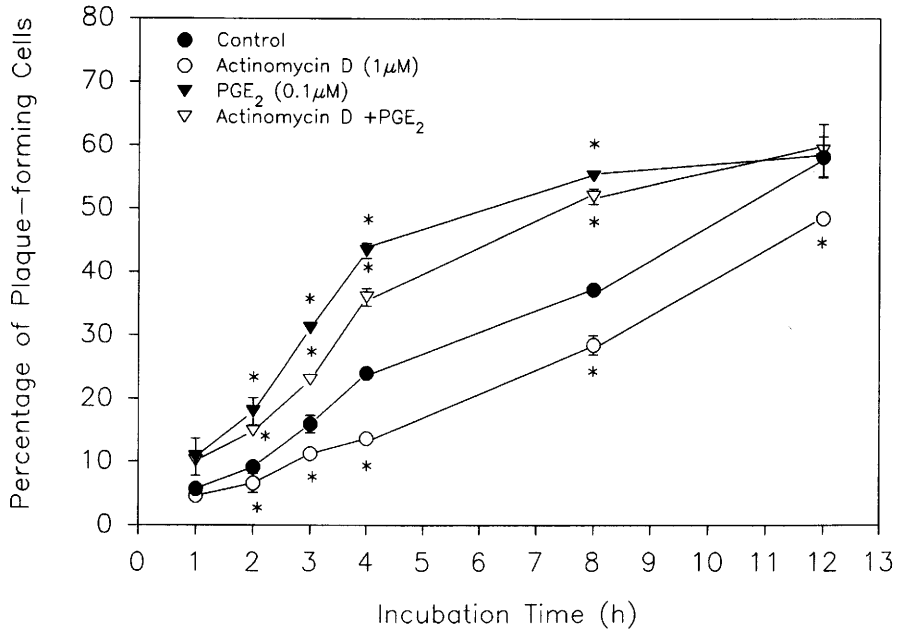

Figure 2. Effect of actinomycin D, PGE_2, or actinomycin D and PGE_2 combined on the rate of plaque formation. The data shown are the mean ± SE from three independent experiments, using cells obtained from Day 33–39 pregnant pigs. Where no error bar is shown, the SE is less than the size of the symbol. Values denoted by an asterisk are significantly different than control values ($P < 0.05$). There was no significant difference between the percentage of plaque-forming LLC in monolayers exposed to PGE_2 alone or to actinomycin D + PGE_2 (inverted triangles) at any time point.

that the ability of LLC to successfully form plaques in the presence of inhibitors of protein synthesis is attributable to the mobilization of relaxin from older, stored hormone that occurs even in the absence of stimulation. On the other hand, the rate of relaxin release by relaxin-releasing LLC was significantly diminished by cycloheximide and actinomycin D treatment, evidence that LLC also utilize newly formed relaxin synthesis for basal release. A small fraction of these LLC (approximately 10%) may be wholly dependent on newly synthesized hormone, since cycloheximide appeared to prevent relaxin release from these cells. These conclusions are consistent with the prior report (in which an identical experimental paradigm was used) which showed that growth hormone- and prolactin-releasing populations of the rat anterior pituitary exhibit the same secretory characteristics (3), and raise the possibility that these phenomena may be of general relevance.

Nevertheless, while cycloheximide and actinomycin D have been widely exploited in the investigation of hormone synthesis, they are severe treatments that result in a general suppression of protein synthesis in treated cells. Thus, although the maximally effective doses of inhibitors (with respect to relaxin release) were similar or less than those used in previously published studies of hormone release (3, 9–10), alternate interpretations of the present data are conceivable. For example, it is a possibility that both inhibitors may have blocked the synthesis of proteins involved in the synthesis and trafficking of relaxin, or associated with the packaging or transport of relaxin granules, or implicated in autocrine ligand-receptor interactions. We cannot positively exclude these possibilities, although the observation that cycloheximide- and actinomycin-treated LLC, when stimulated by PGE_2, released relaxin at significantly increased rates that were comparable to non-inhibitor-treated LLC suggests that the pathways associated with release of stored relaxin were not compromised by inhibitor treatment.

The physiological significance of newly synthesized and stored relaxin to basal release by LLC remains to be defined. One obvious possibility, however, is that the relative contribution of these two pathways to relaxin release, at the level of the individual cell, is implicated in the strikingly heterogenous nature of relaxin release (7, 8). It is also intriguing that basic fibroblast growth factor, a growth factor present in porcine luteal tissue (11), inhibited relaxin release (12) in a pattern that was generally similar to that of cycloheximide (present study). This association raises the possibility that basic fibroblast growth factor may inhibit relaxin release, at least in part, via a suppression of new hormone synthesis.

In summary, this study provides evidence to show that the majority of LLC employ a mix of newly synthesized and old, preformed relaxin to achieve basal release, but that a subset of LLC appears to be wholly dependent on newly synthesized hormone to achieve basal release. Investigation of the relative activity of

these pathways of release will provide a potentially useful approach to better understand the mechanistic basis of heterogenous relaxin release, and the molecular action of relaxin secretagogues.

This work was supported by HD-22786 (M. J. T.). The authors thank Dr. David Sherwood, University of Illinois, Urbana-Champaign, for the generous donation of porcine relaxin antiserum.

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