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Role of the degradation process in the mitogenic effect of epidermal growth factor

(mitogen degradation/leupeptin/granulosa cells/cell proliferation/DNA synthesis)

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ABSTRACT **The protease inhibitor leupeptin inhibits the degradation process of ^{125}I-labeled epidermal growth factor (^{125}I-EGF) by cultured bovine granulosa cells. At 80 μg/ml, leupeptin inhibited the appearance of degradation products of ^{125}I-EGF in the medium by 95% during 1 hr of incubation and by 90% during 24 hr of incubation when the cells were exposed to 5 ng of ^{125}I-EGF per ml. In contrast, cultures exposed to either saturating (10 ng/ml) or nonsaturating (0.1 ng/ml) concentrations of EGF in the presence of leupeptin (80 μg/ml) exhibited an increase in DNA synthesis that was 70–80% that of cultures exposed to EGF alone. Cultures responded to either EGF or fibroblast growth factor with a logarithmic increase in cell number and, over a period of 8 days, the number of cells increased 10- to 18-fold. Addition of leupeptin did not diminish the growth rate of the cultures. In the presence of leupeptin, ^{125}I-EGF accumulated within the granulosa cells and was in a form that was precipitable with antiserum against EGF and that comigrated on isoelectric focusing with native ^{125}I-EGF. That a full mitogenic response can be obtained despite a 90–95% inhibition of EGF degradation at either saturating or nonsaturating concentrations of the mitogen suggests that a proteolytic degradation of a given mitogen may not be involved in the induction of a proliferative response.**

Cultured bovine granulosa cells derived from medium-sized ovarian follicles are highly responsive to both epidermal (EGF) and fibroblast (FGF) growth factors (1). Carpenter and Cohen (2) have demonstrated in cultured fibroblasts that, after binding of human EGF to specific cell surface receptor sites, the receptor–EGF complexes are internalized and degraded by lysosomal proteases. Similar results have been obtained by Vlodavsky *et al.* (3) in a study on the interaction of EGF with bovine granulosa cells. Although a random distribution of receptor–EGF complexes on the cell surface is observed initially, at 37°C these complexes undergo a rapid aggregation (patching) followed by internalization (4, 5). The importance of such a local aggregation of hormone–receptor complexes for the induction of a mitogenic response was recently demonstrated by Shechter *et al.* (6).

Das and Fox (7) proposed four transduction mechanisms to explain how the EGF–receptor interaction can lead to a production of a second messenger which drives the cell through G_1 and commits it to enter the S phase of the cell cycle. These are: (*i*) stimulation of second messenger production by receptor-mediated catalysis at the cell surface [this stimulation may be the phosphorylation of a specific cell surface protein, as observed by Carpenter *et al.* (8)]; (*ii*) receptor-mediated transport of EGF, which then serves as the second messenger either with or without further modification; (*iii*) proteolytic processing of EGF in lysosomes, leading to the formation of second messenger; (*iv*) proteolytic processing of receptors or some other endocytosed membrane proteins to yield new factors which either can serve as second messenger or have the capacity to produce one. However, to date no regulatory action of a protein hormone has been shown to require hormone degradation (9) and it is not even clear whether the internalization, with or without degradation, is required in order to elicit an effect on cell proliferation and DNA synthesis or whether it is simply needed to inactivate the mitogen.

In the present study, the protease inhibitors leupeptin and antipain, which specifically inhibit the lysosomal protease cathepsin B (10, 11), have been used to evaluate the role of degradation in the induction of cell proliferation by EGF. Our results demonstrate that an inhibition of the lysosomal degradation of ^{125}I-labeled EGF (^{125}I-EGF) has no effect on the proliferative response of cultured bovine granulosa cells to that mitogen.

MATERIALS AND METHODS

Materials. FGF was purified from bovine brains as described (12). EGF was purified as described by Savage, Cohen, and coworkers (13, 14). Iodination of EGF was performed according to the procedure of Hunter and Greenwood (15) as modified by Vlodavsky *et al* (3). The EGF specific activity was 3–4 × 10^5 cpm/ng. Na^{125}I was obtained from Amersham/Searle; [*methyl*-^{3}H]thymidine was from New England Nuclear; tissue culture medium (Ham's F-12) was from GIBCO; calf serum was from Irvine Scientific; gentamycin was from Schering; tissue culture dishes were from Falcon; Ampholine pH 4–6 was from Bio-Rad; and Ampholine pH 3.5–10 was from LKB; leupeptin and antipain were kindly provided by M. Umezawa and were purchased from Peptide Institute, Osaka, Japan. Rabbit anti-EGF antiserum was prepared as described by Byyny *et al.* (16). When tested by quantitative precipitin tests, at equivalence 0.1 ml of rabbit EGF antiserum precipitated 3 μg of EGF.

Establishment and Maintenance of Primary Granulosa Cell Cultures. Primary granulosa cell cultures were established from small-sized follicles (4–6 mm diameter) as described (17). The granulosa cells were seeded (1 × 10^5 cells per 10-cm dish) in 10 ml of Ham's F-12 medium supplemented with 10% calf serum. FGF (50 ng/ml) or EGF (10 ng/ml) was added every other day. Within a week the cells reached confluence and were passaged at a split ratio of 1:50. Passages 2–6 were used in this study. The cells were subcultured in 35-mm dishes in 2 ml of Ham's F-12 medium containing 10% calf serum at a density of 1, 4, and 5 × 10^4 cells per dish for the study of growth-rate kinetics, [^{3}H]thymidine incorporation, and EGF binding and degradation, respectively.

Double Immunoprecipitation of ^{125}I-EGF. The tissue culture medium was collected, and the monolayer was washed 10 times with phosphate-buffered saline and extracted with 1.0

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Abbreviations: EGF, epidermal growth factor; FGF, fibroblast growth factor.

ml of 10 mM Tris, pH 8.0/0.1% $NaDodSO_4$/0.5% Triton X-100/1 mM phenylmethylsulfonyl fluoride. Insoluble material was removed from the collected medium and lyzed cells by centrifugation (10,000 × *g*, 5 min, 4°C). Three microliters of rabbit anti-EGF antiserum was added to 1 ml of medium or lyzed cells and the samples were incubated for 2 hr at 37°C; then 10 μl of goat anti-rabbit immunoglobulin antiserum (35% ammonium sulfate cut) was added. The samples were incubated for 1 hr at 37°C and then for 16 hr at 4°C. The precipitates were sedimented by centrifugation at 10,000 × *g* for 5 min at 4°C. The supernatant was carefully aspirated and the pellet was washed once. The radioactivity present in both the supernatant and pellet was measured. Preliminary tests with the rabbit anti-EGF antiserum yielded a precipitation value of 96.1% in freshly labeled EGF versus 70% after 1 month of storage at −70°C. Therefore, only preparations of freshly labeled ^{125}I-EGF or of ^{125}I-EGF reseparated on Sephadex G-10 were used.

RESULTS

Effect of Leupeptin on the Rate of Degradation of ^{125}I-EGF. In order to measure the release of cell-associated ^{125}I-EGF, confluent monolayers of granulosa cells were exposed to ^{125}I-EGF (5 ng/ml, 1 hr, 37°C) and then washed free of unbound ^{125}I-EGF. After an additional 1 hr of incubation at 37°C, the radioactivity present in the medium was measured. Prior exposure of confluent granulosa cell monolayers to the proteases inhibitor leupeptin for 16 hr inhibited the release into the medium of cell-bound ^{125}I-EGF in a concentration-dependent manner (Fig. 1). In the absence of proteases inhibitor, 80–90% of the cell-bound ^{125}I-EGF was released into the medium within the first hour of incubation. In contrast, if the cultures were first exposed for 16 hr to saturating concentrations of leupeptin, the release of cell-bound ^{125}I-EGF into the medium decreased by 75–80% and did not increase beyond the first hour of incubation. After a 3-hr incubation, as much as 75–80% of the initial cell-bound EGF was still associated with the cells (Fig. 1). At 30 μg/ml, leupeptin only partially inhibited the release of cell-bound ^{125}I-EGF, so that at the end of a 3-hr incubation the amount of cell-associated ^{125}I-EGF was almost the same as that found after 1 hr in the absence of the inhibitor. The protease inhibitor antipain was found to inhibit the ^{125}I-EGF degradation to the same extent as leupeptin (unpublished results).

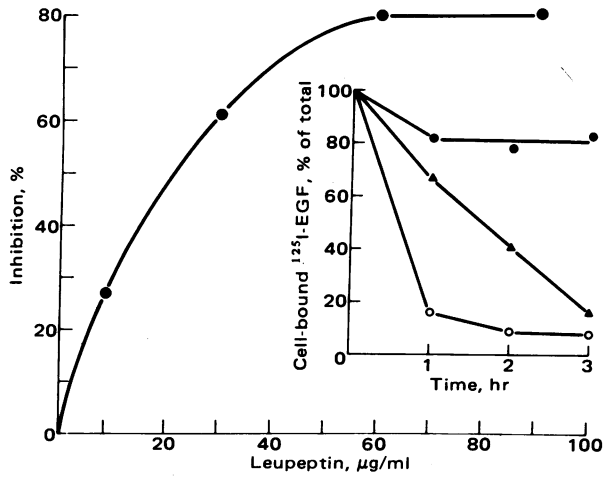

FIG. 1. Effect of leupeptin on the release of cell-bound ^{125}I-EGF from granulosa cells. Confluent cultures were preincubated in the presence of increasing amounts of leupeptin for 16 hr in fresh Ham's F-12 medium containing 10% calf serum, and then ^{125}I-EGF (5 ng/ml, 1.5 × 10^6 cpm per dish) was added. After 1 hr at 37°C, the unbound radioactivity was removed by washing the cells 10 times with cold (4°C) phosphate-buffered saline containing 0.1% albumin. Fresh medium (1 ml) was added and the dishes were incubated for 1 hr at 37°C. At the end of the degradation period the cultures were washed with phosphate-buffered saline. The cells were then dissolved in 0.1 M NaOH, and the radioactivity was measured with a Beckman 310 series gamma counter. The results were expressed as the relative percentage inhibition of release of radioactivity from the cells. (*Inset*) The cells were preincubated for 16 hr in the presence of leupeptin at 30 μg/ml (▲) or 80 μg/ml (●) and in its absence (○). The degradation of the ^{125}I-EGF that bound to the cells was then followed as a function of time. The results are expressed as the relative percentage of cell-bound radioactivity remaining at the indicated times, taking as 100% the amount of radioactivity bound to the cells initially.

The nature of the cell-bound ^{125}I-labeled material released into the incubation medium was analyzed by double immunoprecipitation using a monospecific rabbit anti-EGF antiserum (Table 1). Granulosa cells that had been allowed to bind and internalize ^{125}I-EGF released, during a 1-hr incubation, about 80% of the initial amount of cell-associated ^{125}I-EGF into the medium. That labeled material was composed of two distinct fractions. One, which amounted to 25% of the released ^{125}I-EGF, was precipitated by double immunoprecipitation with anti-EGF antiserum and the other, which amounted to 75%, was not precipitable (Table 1). The precipitable fraction may represent intact EGF molecules that were released into the medium due to a spontaneous dissociation of EGF from its cell surface receptor sites. The fraction that could not be precipitated represented the actual release of ^{125}I-EGF degradation products. Preincubation of cells in the presence of leupeptin did not affect the spontaneous dissociation of the cell-bound ^{125}I-EGF but greatly reduced the amount of nonprecipitable ^{125}I-labeled material released into the medium. With cells preincubated with leupeptin, less than 5% of the initial amount of cell-associated ^{125}I-EGF was in the form of nonprecipitable ^{125}I. Leupeptin thus inhibited the process of lysosomal degradation of the internalized ^{125}I-EGF by at least 95%. Experiments with sparse granulosa cell cultures (10^5 cells per 35-mm dish) yielded results similar to those obtained with confluent cultures (5 × 10^5 cells per dish) (unpublished results).

In order to analyze the effect of leupeptin on the long-term degradation of ^{125}I-EGF, confluent granulosa cell monolayers that first had been preincubated for 16 hr in the presence or

Table 1. Fate of cell-bound ^{125}I-EGF in the presence or absence of leupeptin, studied by anti-EGF antiserum

Sample	Leupeptin absent		Leupeptin present	
	cpm/dish	% of total	cpm/dish	% of total
Medium				
Supernatant	16,600	65.0	1,784	5.2
Pellet	4,573	17.9	4,622	13.6
Cells				
Supernatant	1,954	7.6	1,187	3.5
Pellet	2,420	9.5	26,422	77.7
Total	25,547	100	34,015	100

^{125}I-EGF (5 ng/ml, 1.5 × 10^6 cpm per dish) was allowed to bind for 1 hr at 37°C to confluent cultures of bovine granulosa cells that had been preincubated for 16 hr in fresh Ham's F-12 medium containing 10% calf serum and in the presence or absence of leupeptin (80 μg/ml). The culture dishes were then washed 10 times with cold (4°C) phosphate-buffered saline containing 0.1% albumin. The cell-associated radioactivity was 22,185 or 31,101 cpm of ^{125}I-EGF in the absence or presence of leupeptin, respectively. One milliliter of fresh medium was added, and the cultures were incubated for 1 hr at 37°C. The medium containing the released radioactivity was collected. The cells were washed with phosphate-buffered saline and lysed in 1 ml of 10 mM Tris, pH 8.0/0.5% Triton X-100, 0.1% $NaDodSO_4$/1 mM phenylmethylsulfonyl fluoride. Double immunoprecipitation of ^{125}I-EGF in both medium and cell layer was performed.

absence of leupeptin (80 μg/ml) were exposed to ^{125}I-EGF (5 ng/ml) for 24 hr. The nature of the radioactive material present in the cell-free medium was then studied both by double immunoprecipitation and by gel filtration of the medium on a Bio-Gel P-10 column. After a 24-hr incubation of freshly iodinated EGF at 37°C in the absence of cells, there was spontaneous breakdown of ^{125}I-EGF so that 10% of the total radioactivity could no longer be precipitated with anti-EGF antiserum or be eluted with intact EGF when applied to a Bio-Gel P-10 column (Table 2). The same degree of spontaneous breakdown was obtained regardless of whether the incubation medium contained serum or leupeptin and could be observed when the ^{125}I-EGF was incubated in the presence of phosphate-buffered saline alone. In terms of actual counts, the spontaneous breakdown during a 24-hr period amounted to about 60% of the total nonprecipitable radioactivity obtained in the presence of cells that actively degraded the internalized ^{125}I-EGF. In contrast, incubation of cells with ^{125}I-EGF in the presence of leupeptin (80 μg/ml) yielded, during the first 12 hr, no breakdown above that obtained in the absence of cells and only about 10% of the value obtained in the absence of leupeptin during a 24-hr incubation period. Because incubation of cells with EGF for 12–24 hr is enough to obtain a maximal effect on thymidine incorporation in either the presence or absence of leupeptin, these results demonstrate that degradation of EGF during that period is not likely to play a critical role. This conclusion was further confirmed when similar results were obtained with cells that were incubated with subsaturating concentrations of EGF.

Nature of the ^{125}I-EGF Internalized by Granulosa Cell Cultures Incubated in the Presence or Absence of Leupeptin. The accumulation of ^{125}I-EGF in granulosa cells exposed to leupeptin is shown in Fig. 2. Cells were preincubated in the presence or absence of leupeptin and exposed for various periods of time to ^{125}I-EGF. The cells were then washed, trypsinized, and spun down, and the radioactivity in both pellet and supernatant was measured. The radioactivity present in the supernatant represents the amount of ^{125}I-EGF bound to the cell surface and therefore accessible for release by trypsin; that remaining in the pellet represents the amount of ^{125}I-EGF internalized by the cells and hence no longer accessible to trypsinization. Preincubation of cells in the presence of leupeptin increased the amount of ^{125}I-EGF that was no longer accessible for trypsinization but had no effect on the amount of ^{125}I-EGF released by trypsin. This amount remained at 20–60 pg of EGF per 10^6 cells throughout the experiment. Thus, after a 24-hr exposure to ^{125}I-EGF, the leupeptin-treated cells contained as much as 12.5-fold more EGF than did untreated cells.

Table 2. Degradation of ^{125}I-EGF in the presence or absence of leupeptin

	12-hr incubation		24-hr incubation	
	Free ^{125}I	Degraded ^{125}I-EGF	Free ^{125}I	Degraded ^{125}I-EGF
Saline	—	—	146,140	—
Cells + unlabeled EGF in excess	143,160	—	147,880	—
Cells	184,150	40,990	224,980	77,100
Cells + leupeptin	142,800	0	155,498	7,618

Freshly prepared ^{125}I-EGF (5 ng/ml, 1.5 × 10^6 cpm per dish) was added to confluent cultures of bovine granulosa cells that had been preincubated in the presence or absence of leupeptin (80 μg/ml) for 16 hr in Ham's F-12 medium containing 10% calf serum. To measure the nonspecific degradation of ^{125}I-EGF, excess unlabeled EGF (2 μg/ml) and ^{125}I-EGF were added together to cultures. To measure the spontaneous breakdown of ^{125}I-EGF, the mitogen was incubated in phosphate-buffered saline alone. After incubations of 12 and 24 hr at 37°C, the media were collected, and double immunoprecipitation using anti-EGF antiserum was performed. Alternatively, the media were submitted to gel filtration on a Bio-Gel P-10 column as described (2). The amount of free ^{125}I determined by both techniques was the same and the mean value is presented in the table. The amount of degraded ^{125}I-EGF was expressed as the difference between the amount of free ^{125}I in the presence or absence of leupeptin and in the presence of unlabeled EGF in excess.

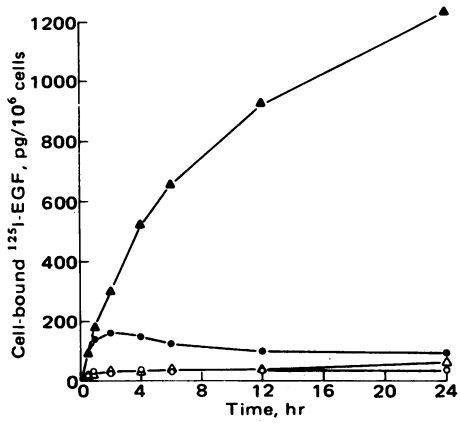

FIG. 2. Time-course of ^{125}I-EGF binding to granulosa cells in the presence (▲, △) or absence (●, ○) of leupeptin at 80 μg/ml. The cells were preincubated for 16 hr in the presence or absence of leupeptin and then were exposed to ^{125}I-EGF for various periods of time. Cultures were then washed 10 times with phosphate-buffered saline and treated with 0.25% trypsin (0.75 ml, 10 min, 37°C). Trypsinization was stopped by addition of Ham's F-12 medium (0.75 ml) containing 10% calf serum. The cell suspension was centrifuged (10,000 × *g*, 3 min). The supernatant was collected for assay of radioactivity (○, △); the pellet was washed once and then assayed (●, ▲). The experiment was done in duplicate.

The nature of the ^{125}I-EGF accumulated within the granulosa cells was studied by using isoelectric focusing. When unlabeled EGF was applied to an isoelectric focusing gel, a single band was obtained at pH 4.5. In contrast, electrophoresis of iodinated EGF yielded three peaks at the range of pH 4.5, which possibly represent different degrees of iodination of the EGF molecules. Similarly, a sample of lysed cells that had been exposed for 24 hr to ^{125}I-EGF in the presence of leupeptin (80 μg/ml) gave three peaks that comigrated with the intact ^{125}I-EGF (Fig. 3). In cultures exposed to ^{125}I-EGF for 1 hr followed by 1 hr of degradation in the absence of leupeptin, no more than 10% of the total cell-associated ^{125}I-EGF was precipitated by the anti-EGF antiserum, whereas as much as 91% of the total cell-associated ^{125}I-EGF was specifically immunoprecipitated in cultures that were preincubated with leupeptin and exposed to ^{125}I-EGF (Table 1). After 24 hr of accumulation, 90% of the cell-associated radioactivity was precipitated by anti-EGF antiserum. These results demonstrate that, in the presence of protease inhibitors, EGF accumulates inside the cells as an intact molecule.

Effect of Leupeptin and Antipain on Cell Proliferation and Initiation of DNA Synthesis Induced by EGF or FGF. The proliferation of granulosa cells was totally dependent upon the addition to the medium of either FGF or EGF (Fig. 4). After the addition of either FGF or EGF, logarithmic cell proliferation resumed. Leupeptin (80 μg/ml) did not affect the survival of the granulosa cells maintained in the presence of F-12 medium supplemented with either 1% or 10% calf serum. More importantly, it slightly increased the rate of cell proliferation in cultures maintained in the presence of 1% calf serum and exposed to EGF. Cultures maintained in the presence of 10%

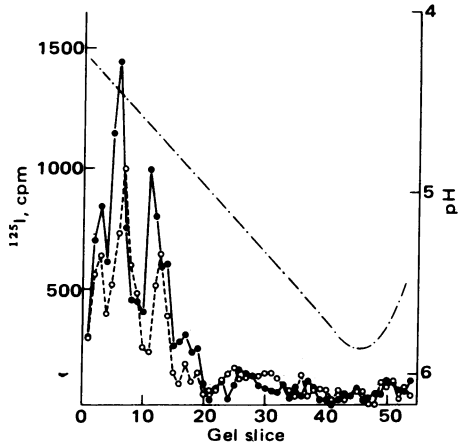

FIG. 3. Pattern of ^{125}I on isoelectric focusing gels of native ^{125}I-EGF (○) and cell-bound ^{125}I-EGF in the presence of leupeptin (●). Granulosa cells were allowed to bind ^{125}I-EGF (5 ng/ml, 1.5×10^6 cpm per dish) for 24 hr in the presence of leupeptin at 80 μg/ml. At the end of the binding period, the cells were lysed in 0.1 ml of lysis buffer (18) and applied to a nonequilibrium pH gradient gel electrophoresis containing pH 4–6 and 3.5–10 Ampholines at a ratio of 4:1. The gels were run at 450 V for 6 hr and then cut into 1-mm slices and assayed in a Beckman series 310 gamma counter. The pH gradient (----) was determined by cutting a gel into 3-mm pieces and measuring the pH. For comparison (○), a sample of native ^{125}I-EGF was run. Native ^{125}I-EGF (12,000 cpm) and the lysate of cells treated in the presence of leupeptin (18,000 cpm) were applied to each gel.

calf serum and exposed to either EGF or FGF showed a slightly lower rate of growth in the presence than in the absence of leupeptin.

The stimulation of DNA synthesis was studied by measuring the incorporation of [^{3}H]thymidine into DNA (Fig. 5). Granulosa cells maintained in 1% calf serum showed a low degree of thymidine incorporation. Addition of EGF to a final concentration of 0.1 or 10 ng/ml caused a 9- or 29-fold increase, respectively, in thymidine incorporation. The addition of either leupeptin (80 μg/ml) or antipain (80 μg/ml) to cultures maintained in the presence of EGF (10 ng/ml) caused a 25% decrease in thymidine incorporation. When cultures were exposed to FGF (50 ng/ml), the addition of leupeptin (80 μg/ml) decreased the response, in terms of thymidine incorporation, by 9%. Addition of leupeptin (80 μg/ml) or antipain (80 μg/ml) to cultures maintained in the presence of a nonsaturable concentration of EGF (0.1 ng/ml) resulted in no more than a 30% decrease in thymidine incorporation as compared to cultures maintained in the absence of protease inhibitors. Because, under the same conditions, EGF degradation was inhibited by as much as 90–95%, one would have expected a much higher inhibition of thymidine incorporation if degradation were to play an essential role in mediating the proliferative response induced by EGF.

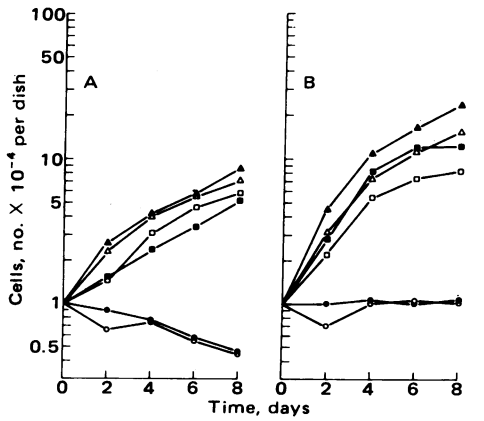

FIG. 4. Effect of EGF or FGF on growth of granulosa cells in the presence or absence of leupeptin. At 8 hr after the cells were seeded, the medium in half of the dishes was replaced with fresh medium containing 1% calf serum (*A*); the rest of the dishes contained 10% calf serum (*B*). At the same time, leupeptin (80 μg/ml) was added to some of the dishes. One day later, and every other day thereafter, EGF (10 ng/ml) or FGF (50 ng/ml) was added. Every other day, duplicate dishes were trypsinized and the cells were counted with a Coulter Counter. The additions were: none (●), leupeptin (○), EGF (■), EGF + leupeptin (□), FGF (▲), and FGF + leupeptin (△).

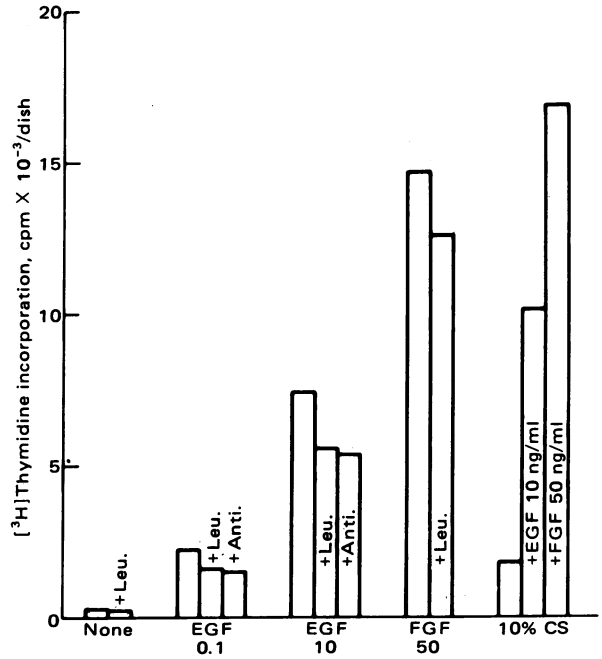

FIG. 5. Effects of leupeptin (Leu.) and antipain (Anti.) on the DNA synthesis stimulated by EGF or FGF in granulosa cells. Granulosa cells were plated at 4×10^4 cells per 35-mm dish. Eight hours later, the medium was changed to a fresh medium containing 1% calf serum (CS). After 48 hr, leupeptin (80 μg/ml) or antipain (80 μg/ml) was added to some of the dishes; after 72 hr, EGF (10 ng/ml), FGF (50 ng/ml), calf serum (10%), or calf serum (10%) plus EGF or FGF was added. Eight hours later, [^{3}H]thymidine was added at a final concentration of 2 μCi/ml to all dishes for 16 hr of incorporation. At the end of the incorporation period, the cells were washed three times with cold (4°C) phosphate-buffered saline (pH 7.4) dissolved in 1 ml of 1% Triton X-100. The contents of the dishes were collected on a Whatman glass-fiber filter and washed three times with 95% ethanol, and their radioactivity was measured with a Beckman LS 8000 scintillation counter. All experiments were performed in triplicate.

DISCUSSION

After binding of ^{125}I-EGF to specific cell surface receptor sites, the receptor–mitogen complexes are internalized and degraded by the lysosomal system. This is followed by the rapid release of radioactive degradation products into the medium (2, 3). The nature of the ^{125}I-labeled material released into the medium was analyzed in the present study by using anti-EGF antiserum and by gel filtration on Bio-Gel P-10 columns. In the short-term degradation experiment, it was found that about 20–25% of the released radioactivity is intact ^{125}I-EGF and 75–80% is degradation products, as described by Carpenter and Cohen (2). This implies that, by inhibiting the degradation process, one cannot expect to inhibit the release reaction by more than 75–80%. Therefore, the 75–80% inhibition of the release reaction observed in the presence of either leupeptin or antipain in fact

represents more than 95% inhibition of the degradation process. However, any molecular explanation for the mitogenic effect of EGF must take into account the observation that EGF must be present in the medium for an extended period of time in order to induce cell proliferation. Leupeptin inhibits the degradation of ^{125}I-EGF by 100% during 12 hr of incubation and by 90% over a 24-hr period. The ^{125}I-EGF that accumulated within the leupeptin-treated granulosa cells was found to be in the form of intact ^{125}I-EGF because 90% of it was precipitated with anti-EGF antiserum and it comigrated with intact ^{125}I-EGF on isoelectric focusing gel. The 10% nonprecipitable ^{125}I radioactivity present in granulosa cells incubated for 24 hr in the presence of ^{125}I-EGF and leupeptin can be explained by a spontaneous breakdown of the iodinated EGF molecule, as was found to occur when ^{125}I-EGF was incubated in phosphate-buffered saline for 24 hr at 37°C. These results suggest that the ^{125}I-EGF is not degraded within cells that are exposed to protease inhibitors.

The mitogenic effect of EGF was investigated by studying either the initiation of DNA synthesis, which reflects the first cell cycle, or the increase in cell number, which reflects the long-term effect of EGF and thereby serves as an absolute criterion for mitogenic activity. At concentrations that inhibited the degradation process, neither leupeptin nor antipain inhibited the proliferation of cells induced by EGF or FGF in cultures maintained with 1% calf serum. With cells maintained in 10% calf serum, there was a slight inhibition of cell proliferation (10%) when both leupeptin and EGF were present, compared to cells exposed to EGF alone. That slight inhibition could be attributed to the accumulation of cytotoxic factors present in serum that are normally degraded by the lysosomal system but that, in the presence of leupeptin, accumulate within the cells. It is also conceivable that such an inhibition by leupeptin is due to an inhibition of proteolytic activity that is normally present in the calf serum and that could potentiate the mitogenic effect of EGF (19). In this regard, leupeptin and antipain are known, in addition to being potent and specific inhibitors of cathepsin B, to inhibit, although to a smaller extent, the activity of plasmin and thrombokinase (10, 11).

That even the slight inhibition of cell proliferation and DNA synthesis caused by the protease inhibitors is not due to the inhibition of EGF degradation is supported by the finding that DNA synthesis induced by nonsaturable concentrations of EGF (0.1 ng/ml) was not inhibited to a higher extent than that induced by saturable concentrations of that mitogen (10 ng/ml). The rate-limiting factor in the stimulation of [^{3}H]thymidine incorporation by EGF at 0.1 ng/ml is the EGF concentration. Therefore, if degradation of EGF is involved in its mitogenic effect, a 90% inhibition of EGF degradation should greatly inhibit the response to EGF. It is more likely that the physiological function of the degradation of EGF by the lysosomal system is merely to destroy the mitogen, so that each molecule can act only once. Shechter *et al.* (20) suggested that the processes of hormone internalization, degradation, and "down regulation" may be irrelevant to the effects of EGF on DNA synthesis. This was based on the observation that, in cultured human fibroblasts, occupation of only a negligible fraction of binding sites that is unwashable might be enough to enhance DNA synthesis (20). This hypothesis was studied with the granulosa cell system and it was found that a simple wash of the monolayer, even 4 hr after the addition of EGF, abolished the incorporation of [^{3}H]thymidine (not shown; performed as described in Fig. 5). Whether or not the internalization process of EGF is relevant to its mitogenic effects on granulosa cells therefore still remains to be studied; however, on the basis of the present results it is reasonable to conclude that the degradation process has little or no role in inducing a mitogenic response of granulosa cells to EGF.

In addition to making it possible to determine the role of the intracellular degradation of mitogens in the proliferative response of granulosa cells, the use of lysosomal inhibitors caused an intracellular accumulation of intact EGF, which will allow us to identify the fate of the cell-associated ^{125}I-EGF within the cells. Preliminary studies (unpublished data) revealed that the cell-surface-bound EGF is translocated into the nucleus and that as much as 15% of the total amount of EGF that had been accumulated in the cytoplasm of inhibitor-treated cells became associated with the nuclei.

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