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# Cytosolic calcium regulates epidermal growth factor endocytosis in rat pancreas and cultured fibroblasts

(cholecystokinin/insulin/calcium ionophore/amylase release)

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**ABSTRACT** **Cholecystokinin octapeptide ($CCK_8$), the COOH-terminal moiety of cholecystokinin (CCK), exerted a rapid inhibitory effect on total cell-associated $^{125}$I-labeled epidermal growth factor ($^{125}$I-EGF) binding by decreasing the rate of EGF internalization in isolated rat pancreatic acini. Removal of $CCK_8$ from incubation medium followed by extensive washing of acini did not abolish its inhibitory effect, indicating that its action was not readily reversible. Proglumide, a competitive antagonist of $CCK_8$, blocked the inhibitory action of the secretagogue. Addition of $CCK_8$ to cells previously exposed to $^{125}$I-EGF did not enhance the release of cell-associated $^{125}$I activity. $CCK_8$ did not inhibit the binding of $^{125}$I-labeled insulin to pancreatic acini. Other pancreatic secretagogues that enhance digestive-enzyme release through $Ca^{2+}$, including caerulein, bombesin, carbachol, gastrin, and the $Ca^{2+}$ ionophore A23187, also inhibited cell-associated $^{125}$I-EGF radioactivity. Further, at 37°C the ionophore A23187 inhibited specific $^{125}$I-EGF binding in human A-431 carcinoma cells, Swiss 3T3 cells, and Rat-1 fibroblasts, and this effect was abolished when $^{125}$I-EGF internalization was reduced by incubating cells at 4°C. It is concluded that alterations in cellular $Ca^{2+}$ in the pancreas and other cells lead to inhibition of EGF endocytosis.**

Epidermal growth factor (EGF) is known to regulate a variety of cellular processes both *in vivo* and *in vitro* (1). It is generally accepted that the first step in the action of EGF is represented by its binding to specific receptors on the cell surface. After binding, the EGF–receptor complexes rotate, cluster together, and enter the cell by a process termed receptor-mediated endocytosis (2). The binding process *per se* activates the signals that induce the rapid biological actions of EGF, whereas receptor clustering appears to be required for induction of DNA synthesis by EGF (3).

As in the case of a number of other polypeptide hormones, high concentrations of EGF decrease the number of EGF receptors on the cell surface through a process called "down-regulation." EGF binding and internalization are also modulated by a variety of nonphysiological agents. Thus, binding of EGF to its receptor is altered by phorbol esters (4), cyclamates (5), quinone derivatives (6), and teleocidine B (7), whereas EGF internalization is inhibited by dansylcadaverine, amantadine, and rimantidine (8). The physiological relevance of these observations is not easily apparent, inasmuch as these compounds are not normally found *in vivo*.

Recently, platelet-derived growth factor (9), fibroblast-derived growth factor (10), the neurohypophyseal hormone vasopressin (11), and cholecystokinin (CCK) (12) have also been shown to inhibit EGF binding. These observations suggest that compounds other than EGF may modulate EGF binding activity *in vivo*. We now report that the inhibitory effect of CCK on EGF binding in isolated rat pancreatic acini may be due to $Ca^{2+}$-mediated inhibition of EGF internalization. In support of this hypothesis, we demonstrate that the $Ca^{2+}$ ionophore A23187 inhibits the internalization of $^{125}$I-labeled EGF ($^{125}$I-EGF) in A-431 human carcinoma cells and other cultured fibroblasts. Inasmuch as a variety of hormones and neurotransmitters are known to act through $Ca^{2+}$, it is possible that EGF internalization may be regulated through similar mechanisms in other cell types.

## MATERIALS AND METHODS

**Preparation of Acini and Cell Culture.** Pancreatic acini were prepared from fasted male Sprague–Dawley rats as reported (13). Freshly isolated acini were incubated for 60 min at 37°C in Hepes-buffered Ringer solution that was titrated to pH 7.40, equilibrated with 100% oxygen (13), and resuspended in fresh Hepes/Ringer buffer (0.5–0.8 mg of protein per ml) for use in experiments. Derivation of Rat-1 fibroblasts (14), A-431 human vulvar carcinoma cells (12), and Swiss mouse 3T3 cells (15) has been described. All cell lines were propagated at 37°C in a 5% $CO_2$/95% air atmosphere in Dulbecco's modified Eagle's medium supplemented with antibiotics and the following serum conditions: 10% newborn calf serum (3T3), 10% calf and 2% newborn calf serum (Rat-1), or 5% newborn calf and 2% fetal calf serum (A-431).

**Binding Studies.** EGF was prepared from mouse submaxillary glands by the method of Savage and Cohen (16) and further purified by reverse-phase high-performance liquid chromatography (14). Biologically active $^{125}$I-EGF (14) and $^{125}$I-labeled insulin ($^{125}$I-insulin) (17) were prepared with chloramine T. To measure binding in acini, 2- to 18-ml suspensions of cells were routinely incubated at 37°C with $^{125}$I-EGF (23.4–53.0 $\mu$Ci/$\mu$g; 1 Ci = 37 GBq) or $^{125}$I-insulin (20.5 $\mu$Ci/$\mu$g). To separate bound and free hormone, 0.5- to 0.7-ml aliquots of cell suspensions were rapidly centrifuged and washed three times with 154 mM NaCl at 4°C (18). Nonspecific binding, determined by incubating acini in the presence of either $^{125}$I-EGF or $^{125}$I-insulin and unlabeled ligand (8 $\mu$g/ml and 50 $\mu$g/ml, respectively), did not exceed 25% of total. To monitor $^{125}$I-EGF internalization, acini and cultured fibroblasts were washed as for binding experiments and incubated for 4 min at 4°C with 0.5 M NaCl (pH 2.5) (8). Cell-associated radioactivity was then assayed separately from radioactivity removed by acid treatment.

Degradation of $^{125}$I-EGF and $^{125}$I-insulin was routinely monitored by precipitation of labeled EGF in incubation medium with trichloracetic acid at 10% final concentration. Degradation products never exceeded 15% of total radioactivity. Supernatant $^{125}$I-EGF was analyzed also by chromatography on a 0.9 × 60 cm column of Sephadex G-50 (fine) that was equilibrated with 4 M urea/1 M acetic acid/0.15 M NaCl/0.2% bovine serum albumin (19). EGF binding studies in cultured cells were performed at either 37°C or 4°C as described (14). The $Ca^{2+}$ ionophore A23187 (Calbiochem) was

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Abbreviations: CCK, cholecystokinin; $CCK_8$, cholecystokinin octapeptide; EGF, epidermal growth factor.

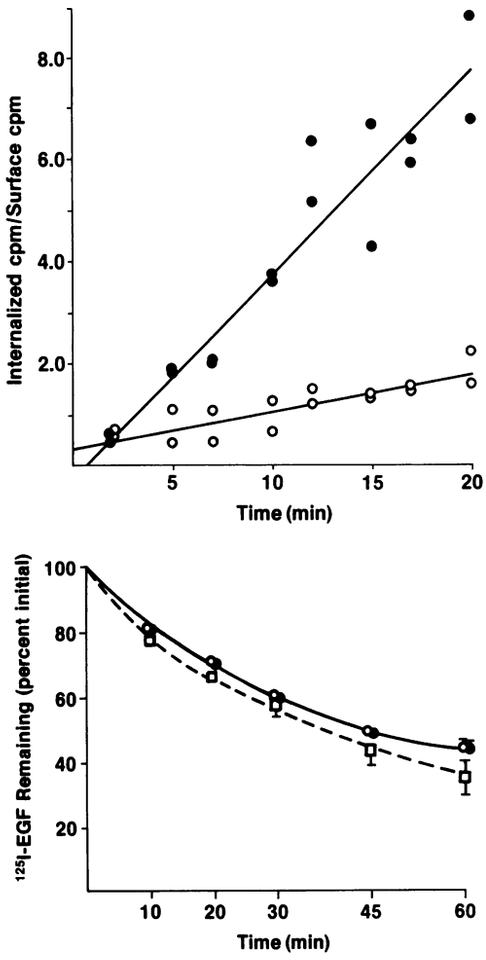


FIG. 1. Internalization and release of $^{125}$I-EGF. Pancreatic acini were incubated at 37°C with 0.24 nM $^{125}$I-EGF. (*Upper*) Acini were incubated for specified times in the absence (●) or presence (○) of 30 nM $CCK_8$. Acini were then treated with 0.5 M NaCl as described. Values are representative of two individual experiments. (*Lower*) Acini were incubated for 60 min with $^{125}$I-EGF, collected, and washed once. Acini were then resuspended in fresh medium without added radioactivity, in the absence (●) or presence of either 0.83 μM EGF (○) or 30 nM $CCK_8$ (□). $^{125}$I-EGF remaining in acini is expressed as the percentage of initial hormone binding. Values are means ± SEM of four experiments.

dissolved in ethanol; the final concentration of ethanol never exceeded 0.5% and did not alter $^{125}$I-EGF binding. Statistical analysis was performed by Student's *t* test. Data were considered statistically significant at $P < 0.05$.

**Amylase Secretion Studies.** To measure amylase secretion, acini were incubated for 30 min at 37°C. Aliquots of cell suspensions (0.5 ml) were centrifuged at 15,000 × *g* for 30 s as previously reported (13), and amylase released into the medium was determined (12). CCK octapeptide ($CCK_8$) was a gift of Miguel Ondetti of Squibb, and proglumide was a gift of Rotta Laboratories (Milan, Italy).

Table 1. Effect of $CCK_8$ on $^{125}$I-EGF and $^{125}$I-insulin binding in pancreatic acini

| Addition | Specific binding, % | |
|---|---|---|
| | $^{125}$I-EGF | $^{125}$I-Insulin |
| None | 6.06 ± 0.80 | 6.07 ± 1.02 |
| $CCK_8$ (30 nM) | 1.03 ± 0.10* | 5.61 ± 0.91 |

Pancreatic acini were incubated for 60 min at 37°C with either $^{125}$I-EGF (0.12 nM) or $^{125}$I-insulin (1.8 nM) in the presence of specified additions. Values represent percentages of radioactivity added and are means ± SEM of three individual experiments.
*$P < 0.005$ when compared with control.

## RESULTS

**Effect of $CCK_8$ on $^{125}$I-EGF Internalization, Dissociation, and Degradation.** We have shown previously that the pancreatic acinar cell possesses specific, saturable, high-affinity EGF receptors and that EGF rapidly internalizes into these cells (18). We also have shown recently that $CCK_8$ exerts a marked inhibitory effect on EGF binding in this cell, which is evident within 5 min of addition of the secretagogue to incubation medium (12). Therefore, we sought to determine whether this inhibition could be due to either decreased influx or enhanced efflux of EGF. To quantitate internalization, the endocytosis rate constant ($k_e$) was calculated from the ratio of intracellular versus surface-bound radioactivity (20). The $k_e$ for the uptake of $^{125}$I-EGF into nonstimulated acini was 0.37 $min^{-1}$ (Fig. 1 *Upper*). $CCK_8$ (30 nM) did not significantly alter the amount of acid-dissociable (cell-surface) radioactivity (data not shown), but markedly decreased the amount of internalized $^{125}$I-EGF, lowering the $k_e$ to 0.066 $min^{-1}$ (mean of two experiments). In contrast, after incubation of acini for 60 min with $^{125}$I-EGF, neither $CCK_8$ nor unlabeled EGF significantly altered the release of cell-associated $^{125}$I radioactivity (Fig. 1 *Lower*). $CCK_8$ did not appear to enhance $^{125}$I-EGF degradation as determined by trichloroacetic acid precipitability and Sephadex chromatography of supernatant radioactivity (data not shown).

**Lack of Effect of $CCK_8$ on $^{125}$I-Insulin Binding in Pancreatic Acini.** We next sought to determine whether $CCK_8$ inhibited the binding of insulin, another polypeptide hormone that is known to rapidly internalize into pancreatic acini (21). Specific binding of $^{125}$I-insulin and EGF was quantitatively similar (Table 1). $CCK_8$ (30 nM) decreased specific, cell-associated $^{125}$I-EGF binding by 83% ($P < 0.005$) but did not significantly alter $^{125}$I-insulin binding.

Table 2. Residual action of CCK on $^{125}$I-EGF binding and amylase secretion in pancreatic acini

| Addition | $^{125}$I-EGF binding, % of control | | Amylase secretion, % of control | |
|---|---|---|---|---|
| | 37°C | 4°C | 37°C | 4°C |
| None | 100 ± 6 | 100 ± 10 | 100 ± 5 | 100 ± 3 |
| EGF (0.83 μM) | 8 ± 2* | 14 ± 3* | 101 ± 3 | 100 ± 1 |
| $CCK_8$ (30 nM) | 40 ± 3* | 51 ± 2* | 869 ± 107* | 854 ± 240† |

Pancreatic acini were incubated for 10 min at either 37°C or 4°C as indicated, washed three times at 23°C or 4°C (respectively) with 20 ml of incubation medium, and resuspended in fresh medium. Acini were then incubated for 30 min at 37°C in the presence of specified additions. Data are expressed as the percentage of respective control values in the absence of either unlabeled EGF or $CCK_8$ and are means ± SEM of three individual experiments.
*$P < 0.005$ when compared with control.
†$P < 0.02$ when compared with control.

Table 3. Lack of effect of proglumide on $^{125}$I-EGF binding in pancreatic acini

| Addition | $^{125}$I-EGF binding, % bound |
|---|---|
| None | 4.68 ± 0.22 |
| EGF (0.83 μM) | 0.99 ± 0.11* |
| Proglumide (10 mM) | 4.57 ± 0.14 |
| Proglumide/EGF | 1.54 ± 0.19* |
| $CCK_8$ (1 nM) | 2.18 ± 0.04* |
| $CCK_8$/proglumide | 4.55 ± 0.22 |

Pancreatic acini were incubated for 60 min at 37°C in the presence of specified additions. Values represent the percentages of radioactivity added and are means ± SEM of three individual experiments. *$P < 0.005$ when compared with control.

**Residual Inhibition of $^{125}$I-EGF Binding.** To determine whether the inhibitory effect of $CCK_8$ on $^{125}$I-EGF binding was reversible, acini were first incubated with 30 nM $CCK_8$ for 10 min at either 37°C or 4°C, washed extensively to remove extracellular $CCK_8$, and resuspended in fresh Hepes/Ringer buffer. $^{125}$I-EGF was then added at the beginning of the second incubation period that was always carried out at 37°C. When acini were first incubated at 37°C, $CCK_8$ inhibited total $^{125}$I-EGF binding by 65% ($P < 0.005$, Table 2) and stimulated amylase release by 869% ($P < 0.001$). Similar effects were observed when acini were first incubated with $CCK_8$ at 4°C and subsequently incubated at 37°C with $^{125}$I-EGF (Table 2). This lack of reversibility suggested that inhibition of EGF binding was dependent on occupancy of the CCK receptor, inasmuch as continued stimulation of amylase secretion is also temperature independent with respect to the first incubation period and correlates with the presence of tightly bound CCK (22). Further, proglumide (10 mM), a competitive antagonist of CCK (23), completely blocked the inhibitory effect of 1 nM $CCK_8$ on EGF binding (Table 3).

**Effect of Other Pancreatic Secretagogues on $^{125}$I-EGF Binding in Pancreatic Acini.** Because most of the biological actions of $CCK_8$ are mediated through $Ca^{2+}$ (24), we next sought to determine whether other pancreatic secretagogues that act through $Ca^{2+}$ also inhibit total $^{125}$I-EGF binding in pancreatic acini. Inhibition of binding was related to the ability of these secretagogues to stimulate amylase secretion (Fig. 2). The decapeptide caerulein and $CCK_8$ were equipotent in inhibiting $^{125}$I-EGF binding and enhancing amylase release. In comparison to $CCK_8$, desulfated $CCK_8$ and bombesin were 1/10th and 1/30th as potent, respectively, with respect to stimulation of amylase release. Both secretagogues were also less effective than $CCK_8$ in inhibiting $^{125}$I-EGF binding. In concert with previous studies (25), the cholinergic analogue carbachol and the gastrin analogue pentagastrin maximally enhanced amylase release at a concentration of 10 μM. Although both secretagogues were equally effective in stimulating amylase release, pentagastrin was less effective than carbachol in inhibiting $^{125}$I-EGF binding. Inhibition of $^{125}$I-EGF binding was also observed with the $Ca^{2+}$ ionophore A23187 (Table 4), an agent that bypasses cell-surface receptors and raises cytosolic $Ca^{2+}$ (24). However, chelation of extracellular $Ca^{2+}$ did not alter the inhibitory effect of $CCK_8$ on total cell-associated $^{125}$I-EGF radioactivity (Table 4).

**Effect of Calcium Ionophore A23187 on $^{125}$I-EGF Binding in Cultured Fibroblasts.** To determine whether alterations in cytosolic $Ca^{2+}$ inhibit $^{125}$I-EGF binding in other cell types, we examined the effect of the calcium ionophore A23187 on $^{125}$I-EGF binding in A-431 human carcinoma cells, Swiss 3T3 mouse fibroblasts, and Rat-1 fibroblasts (Fig. 3). When

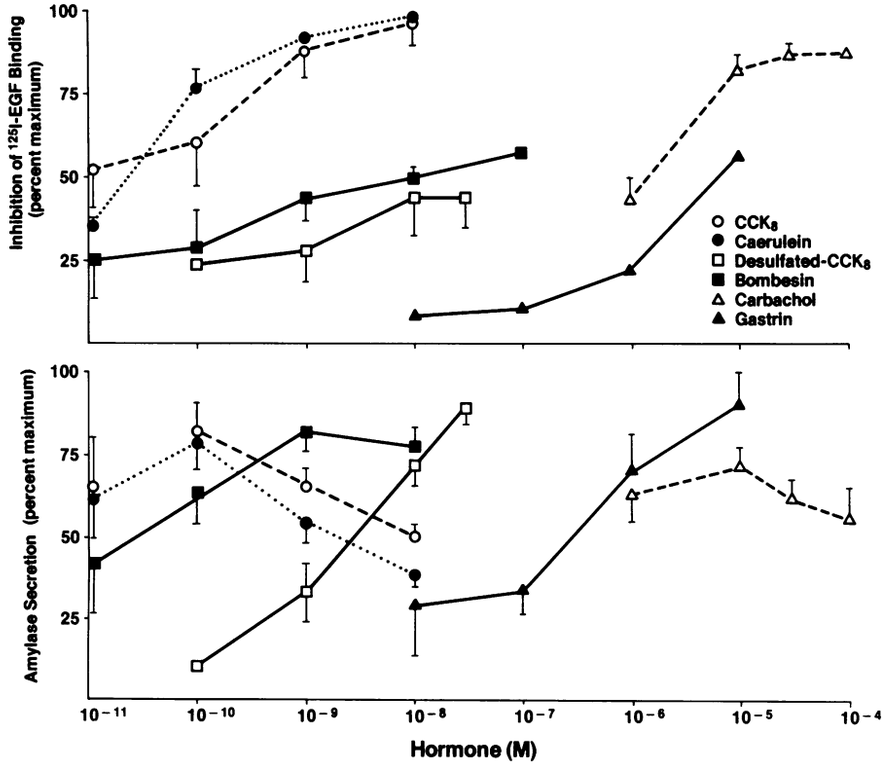


FIG. 2. Relationship of inhibition of $^{125}$I-EGF binding to amylase secretion in pancreatic acini. (*Upper*) Acini were incubated at 37°C for 60 min with $^{125}$I-EGF (0.12 nM) in the presence of specified additions. Values are means ± SEM of three individual experiments, expressed as the percentage of maximal inhibition of specific $^{125}$I-EGF binding in the absence of secretagogues. (*Lower*) Acini were incubated at 37°C for 30 min in the presence of specified additions. Values are means ± SEM of three individual experiments, expressed as the percentage of maximal amylase release by any of the secretagogues during a given experiment.

Table 4. Effect of calcium ionophore A23187 on $^{125}$I-EGF binding in pancreatic acini

| Addition | Concentration, $\mu$M | $^{125}$I-EGF binding, % inhibition |
|---|---|---|
| None | | 0 |
| A23187 | 0.5 | 29 ± 11 |
| | 1 | 34 ± 10* |
| | 5 | 37 ± 10* |
| | 10 | 33 ± 8* |
| | 50 | 65 ± 8† |
| EGTA | 100 | 1 ± 1 |
| $CCK_8$ | 0.03 | 63 ± 5† |
| $CCK_8$/EGTA | | 66 ± 3† |

Pancreatic acini were incubated for 60 min at 37°C with $^{125}$I-EGF (0.12 nM) in the presence of specified additions. Values are means ± SEM of three individual experiments.
*$P < 0.05$ when compared with control.
†$P < 0.001$ when compared with control.

incubations were carried out at 37°C, A23187 inhibited cell-associated $^{125}$I-EGF binding in a dose-dependent manner. Although the ionophore was equipotent in A-431 and 3T3 cells, it was less effective in Rat-1 fibroblasts. When incubations were carried out at 4°C to prevent EGF internalization,

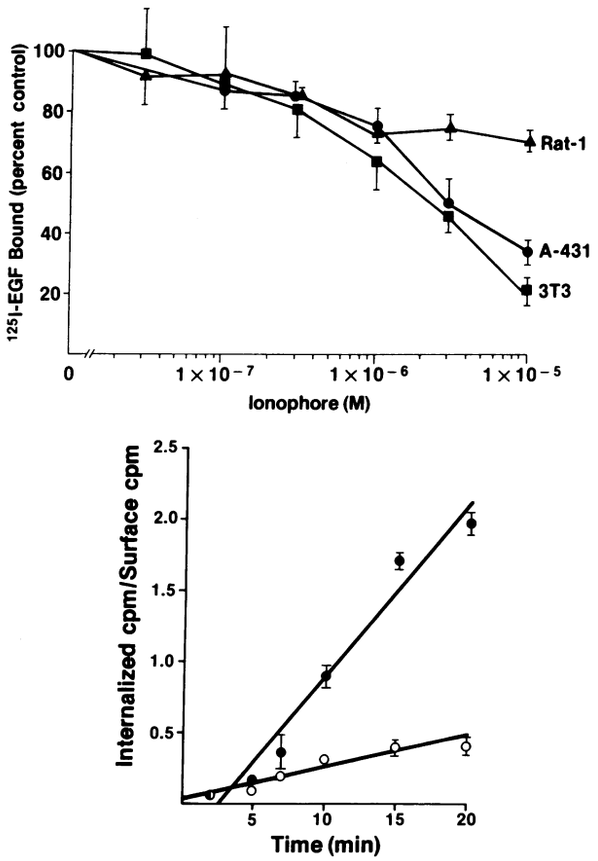


FIG. 3. Effect of $Ca^{2+}$ ionophore A23187 on $^{125}$I-EGF binding in cultured cells. (*Upper*) Cells were plated in 35-mm dishes at a density of either $10^6$ cells per dish (Rat-1 and 3T3 cells) or $5 \times 10^5$ cells per dish (A-431 cells). Cells were washed once just prior to binding studies and incubated at 37°C for 60 min in binding medium (Dulbecco's modified Eagle's medium with 0.1% bovine serum albumin) with 0.17 nM $^{125}$I-EGF and varying concentrations of ionophore. (*Lower*) A-431 cells were incubated as in *A* in the absence (●) or presence (○) of 10 $\mu$M ionophore A23187. Incubations were stopped at specified times, and cells were treated with 0.5 M NaCl as described. Values are means ± SD of specific binding of triplicate plates and are representative of three individual experiments.

Table 5. Temperature dependence of ionophore A23187 inhibition of $^{125}$I-EGF binding in cultured cells

| Cell type | $^{125}$I-EGF binding, % inhibition | |
|---|---|---|
| | 37°C | 4°C |
| 3T3 | 64 ± 8* | 9 ± 2 |
| Rat-1 | 32 ± 3† | 3 ± 3 |
| A-431 | 64 ± 1* | 15 ± 7 |

Cultured cells were incubated with $^{125}$I-EGF (0.17 nM) for 60 min at 37°C or for 120 min at 4°C, in the presence or absence of ionophore A23187 (10 $\mu$M). Values represent the percentage inhibition of specific $^{125}$I-EGF binding with respect to binding in the absence of ionophore and are means ± SEM of three individual experiments.
*$P < 0.001$ when compared with control.
†$P < 0.02$ when compared with control.

the inhibitory effect of A23187 was abolished in all three cell lines (Table 5). Further, the ionophore exerted a marked inhibitory effect on the $k_e$ for the uptake of $^{125}$I-EGF into A-431 cells (Fig. 3 *Lower*). In three experiments with A-431 cells, the ionophore reduced the $k_e$ by 66 ± 5% (mean ± SEM). A smaller inhibitory effect was obtained with Rat-1 fibroblasts (data not shown). The $k_e$ was not examined in 3T3 cells because of low specific binding at early time points.

## DISCUSSION

The present data suggest that the inhibitory effect of CCK on EGF binding in pancreatic acini is due to inhibition of EGF internalization. It is not clear whether this inhibition is associated with alterations in the number or affinity of the acinar cell EGF receptors. However, it is unlikely that CCK inhibits EGF internalization by enhancing the rate of EGF release from acini, or its degradation in incubation medium. It is also unlikely that inhibition of EGF internalization is secondary to stimulation of the secretory process *per se*, inasmuch as vasoactive intestinal polypeptide enhances amylase release but does not inhibit EGF binding in either rat or mouse pancreatic acini (12).

We have reported previously that CCK does not inhibit EGF binding in cell lines that do not possess a CCK receptor (12). This observation suggests that CCK does not inhibit EGF internalization in acini by occupying the EGF receptor. Instead, three lines of evidence indicate that inhibition of EGF internalization is dependent on CCK receptor occupancy. First, removal of CCK from incubation medium did not abolish its inhibitory action, indicating that continued occupancy of the CCK receptor by tightly bound CCK leads to residual inhibition of EGF internalization (22). Second, proglumide, a competitive antagonist of CCK (23), blocked the inhibitory action of CCK. Third, caerulein, desulfated-$CCK_8$, and pentagastrin inhibited $^{125}$I-EGF binding in parallel to their known potencies with respect to stimulation of amylase release and inhibition of $^{125}$I-CCK binding in pancreatic acini (25).

Our present findings suggest that CCK inhibits EGF internalization by mobilizing intracellular $Ca^{2+}$ stores. Thus, the dose–response curve for CCK-mediated inhibition of $^{125}$I-EGF binding paralleled its known effects on stimulation of $Ca^{2+}$ efflux (24); chelation of extracellular $Ca^{2+}$ with EGTA did not block the inhibitory effect of CCK; carbachol and bombesin, compounds that are known to regulate pancreatic enzyme secretion through mobilization of intracellular $Ca^{2+}$ but that bind to receptors that are distinct from the CCK receptor (26), also inhibited EGF binding; and the $Ca^{2+}$ ionophore A23187, an agent that increases cytosolic $Ca^{2+}$ in the pancreas by enhancing both $Ca^{2+}$ influx and $Ca^{2+}$ release from intracellular stores (24), mimicked the effects of $CCK_8$ on EGF binding. In concert with our findings in pancreatic acini, the $Ca^{2+}$ ionophore A23187 inhibited EGF binding in three distinct cultured cell lines (A-431 cells, Rat-1 fibro-

blasts, and 3T3 cells) at 37°C but not at 4°C, a temperature that is known to inhibit EGF internalization (11). Further, the ionophore directly inhibited the rate of endocytosis of $^{125}$I-EGF in two of these cell lines. These findings suggest that alterations in cytosolic $Ca^{2+}$ may inhibit EGF internalization in a variety of cells.

It is not clear whether EGF internalization is inhibited directly by a rise in cytosolic $Ca^{2+}$ or indirectly by the mobilization of cellular $Ca^{2+}$ *per se*. Further, the molecular mechanisms whereby alterations in cytosolic $Ca^{2+}$ might inhibit EGF internalization are not easily apparent. It is possible that increases in cytosolic $Ca^{2+}$ alter microtubule function, thereby interfering with the translocation of the EGF–receptor complex within the cell. However, colchicine, an agent known to disrupt microtubules, does not inhibit EGF binding in either pancreatic acini (unpublished observations) or Swiss 3T3 cells (27). Changes in cytosolic $Ca^{2+}$ also may interfere with the rotational translocation and clustering of the EGF–receptor complex (2), or with other cellular processes. For example, amantadine and other inhibitors of EGF endocytosis have been shown recently to alter phospholipid metabolism in a variety of cells (28), and CCK is known to effect phospholipid metabolism in the pancreas (29). This may explain why several secretagogues (desulfated $CCK_8$, bombesin, and pentagastrin) were not as effective as $CCK_8$ in inhibiting $^{125}$I-EGF binding but enhanced amylase release to the same extent as $CCK_8$. Our findings also indicate that the action of CCK on EGF endocytosis is not necessarily valid for other polypeptide ligands, inasmuch as $CCK_8$ did not inhibit insulin binding in pancreatic acini. Nonetheless, they point to a previously unrecognized mechanism whereby a variety of ligands that act through $Ca^{2+}$ may inhibit the internalization of EGF in target tissues, and thereby conceivably modulate some of its actions.

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