Epidermal growth factor: intracellular Ca^{2+} inhibits its association with pancreatic acini and A431 cells

Craig D. Logsdon and John A. Williams*

Cell Biology Research Laboratory, Mount Zion Hospital and Medical Center, PO Box 7921, San Francisco, CA 94120 and **The Departments of Physiology and Medicine, University of California, San Francisco, CA 94143, USA*

Received 28 October 1983

The uptake of ^{125}I-labeled epidermal growth factor (^{125}I-EGF) by mouse pancreatic acini was inhibited (40–50%) by the secretagogue cholecystokinin octapeptide (CCK_8). Analysis of competitive binding data showed that the apparent K_d of EGF binding increased 135% while the binding capacity was only slightly altered (30% increase). That the effect of CCK_8 on acini was mediated by intracellular Ca^{2+} was indicated by the following: (i) Inhibition of ^{125}I-EGF binding to acini was dose-dependent and paralleled the known abilities of CCK_8, its analogs, and the cholinergic secretagogue carbachol to induce Ca^{2+} efflux from acini; and (ii) addition of the Ca^{2+} ionophore A23187 also inhibited ^{125}I-EGF binding. In addition, EGF association with A431 cells was also inhibited by A23187 in the presence but not the absence of Ca^{2+}.

EGF *Calcium* *Receptor* *Pancreas*

1. INTRODUCTION

We and others have recently reported that pancreatic acini possess EGF receptors [1,2] and that EGF promotes the maintenance of differentiated acinar cell function in culture [1,3]. In the course of these studies we found that cholecystokinin (CCK), a polypeptide hormone structurally unrelated to EGF, inhibited the uptake of ^{125}I-EGF by these cells. Uptake or association of ^{125}I-EGF with cells or membrane will by convention be referred to as 'binding'. This is used as an operational definition and does not indicate the state of cell-associated hormone. A preliminary report of these results has appeared in the proceedings of the 74th Annual Meeting of the American Society of Biological Chemists (Fed. Proc. 42, 1905). All of the major effects of CCK are believed to be mediated by an increase in free intracellular Ca^{2+} [4–6]. Here, we have therefore investigated the inhibitory effect of CCK on ^{125}I-EGF binding to pancreatic acini to determine whether this effect is also mediated by intracellular Ca^{2+}. In addition, we have studied A431 human epidermal carcinoma cells, a model cell type for studying EGF receptors.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Isolated mouse pancreatic acini from fed adult male Swiss Webster mice were prepared and cultured as in [1]. Acini were incubated as suspension cultures in Waymouths medium containing 20 mM Hepes buffer, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.2 mg/ml soybean trypsin inhibitor, and 20% fetal bovine serum at 37°C. All experiments on acini were conducted after 24 h of culture.

A431 cells were propagated in DME-H21 medium containing 10% heat inactivated fetal bovine serum, penicillin, and streptomycin, at 37°C.

2.2. *Binding studies*

EGF (Collaborative Research Inc., receptor grade) was iodinated by a version of the

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

chloramine-T method [1]. Pancreatic acini (0.1–0.2 mg acinar protein/ml) were washed free of culture medium and resuspended in a Hepes-buffered Ringer (HR) enriched with minimal Eagle's medium amino acid supplement, soybean trypsin inhibitor (0.1 mg/ml), 0.5% bovine serum albumin, and gassed with 100% O_2. ^{125}I-EGF (40–60 pM) plus various concentrations of unlabeled hormones and secretagogues were added to the suspension and incubated in a shaking water bath at 37°C. At specific times triplicate 1-ml samples of the suspension were removed and centrifuged at 300 × *g* for 2 min at 4°C. The supernatant was sampled to determine total counts, and the pellets were washed twice with 0.15 M NaCl at 4°C. Radioactivity associated with the washed acinar pellets was measured in a gamma scintillation counter, and the acinar protein measured [7]. Non-specific binding was determined in the presence of an excess of unlabeled EGF (100 nM). Competitive inhibition of specific ^{125}I-EGF uptake by various concentrations of unlabeled EGF was analyzed by fitting plots of bound hormone *vs* free hormone with a non-linear least squares computer program which calculated the affinity and capacity of the EGF binding site [8].

For binding to A431 cells, cells were plated onto 35-mm dishes at 1 × 10^5 cells/dish 48 h prior to the beginning of the experiment. Culture medium was replaced with 2 ml of HR pre-warmed to 37°C and ^{125}I-EGF, non-radioactive EGF, and secretagogues were added at various concentrations. Plated cells were incubated at 37°C for 1 h. The dishes were then washed twice in ice-cold 0.15 M saline and the cells removed by scraping into 1 ml of 0.1 N NaOH in saline. Cell-bound radioactivity was determined and the protein content of each sample (50–100 μg) was measured.

Degradation of labeled hormone in the medium was determined by precipitation of binding supernatants with trichloroacetic acid added at a final concentration of 10%.

3. RESULTS

3.1. *Effects of CCK_8 on EGF binding to pancreatic acini*

Cultured acini incubated with 50 pM ^{125}I-EGF at 37°C for 2 h bound an average of 13.6 ± 1.3% (n = 10) of total radioactivity per mg protein, while non-saturable binding represented only 4% of total binding (0.6 ± 0.1% bound/mg protein). The effect of increasing concentrations of CCK_8 or EGF on the specific binding of ^{125}I-EGF to pancreatic acini is shown in fig.1. CCK_8 inhibited EGF binding in a dose-dependent manner with significant inhibition present at 100 pM and a maximal inhibition of 42.2 ± 2.6% (n = 6) occurring with 3 nM CCK_8. Higher concentrations of CCK_8 did not further inhibit ^{125}I-EGF binding. By contrast, inhibition of ^{125}I-EGF binding by non-radioactive EGF increased with increasing concentrations until inhibition was maximal at 100 nM. CCK_8 had no effect on non-specific ^{125}I-EGF binding determined in the presence of 100 nM EGF. Dibutyryl cGMP, a competitive antagonist of CCK [9], had no effect on specific or non-specific EGF binding when tested alone, but completely blocked the CCK-induced inhibition of EGF binding.

To test whether CCK_8 increased the degradation of ^{125}I-EGF, we measured the trichloroacetic acid precipitability of the ^{125}I-EGF in the binding medium. Equal low levels of degradation averaging 4.4 ± 0.5% (n = 8) were found after 2 h of incubation with acini in the presence or absence of 10 nM CCK_8.

3.2. *Dose-response for inhibition of EGF binding by other Ca^{2+}-mediated secretagogues*

Other Ca^{2+}-mediated secretagogues were tested for their effects on ^{125}I-EGF binding. Fig.2 shows

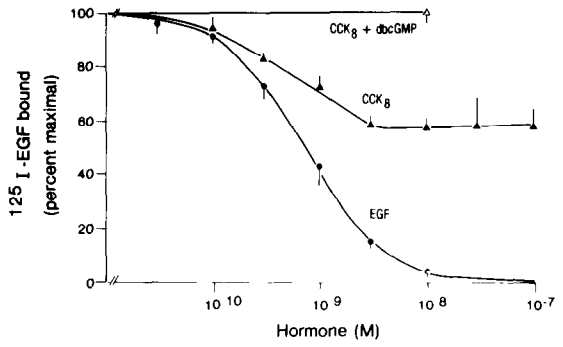

Fig.1. Effects of various concentrations of EGF, CCK_8 and CCK_8 plus 1 mM dbcGMP on the specific binding of ^{125}I-EGF to pancreatic acini. Binding was carried out at 37°C for 2 h after simultaneous addition of tracer and EGF (●), CCK_8 (▲), or CCK_8 plus 1 mM dbcGMP (△). Results are plotted as a % of control value and are the means ± SEM of 6 experiments.

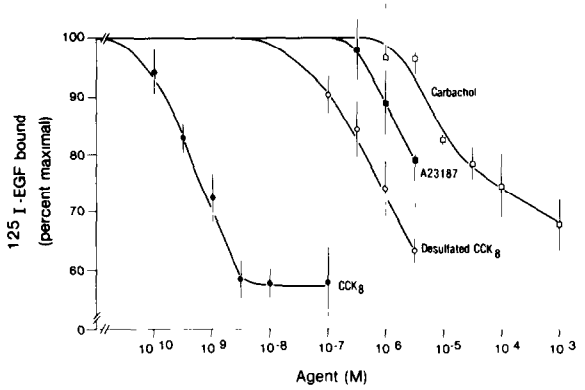

Fig.2. Inhibition of specific ^{125}I-EGF binding to acini by scretagogues whose actions are mediated by calcium. Acini (0.1–0.2 mg protein/ml) were incubated at 37°C for 2 h in the presence of 50 pM ^{125}I-EGF and varying concentrations of CCK_8 (●), desulfated CCK_8 (○), A23187 (■), or carbachol (□). Results are plotted as a % of control value and are the means ± SEM of 6 experiments.

that CCK_8, desulfated CCK_8 and carbachol inhibited the association of ^{125}I-EGF with acini. Desulfated CCK_8 is 200-times weaker than CCK_8, in concert with its known ability to bind to the CCK receptor on pancreatic plasma membranes [10]. Thus inhibition occurred over concentration ranges paralleling the known ability of these secretagogues to mediate Ca^{2+} efflux from pancreatic acini and stimulate amylase release. The Ca^{2+} ionophore A23187, which by-passes receptors but mimics the effects of these secretagogues on acinar cell function by directly increasing intracellular Ca^{2+}, also inhibited ^{125}I-EGF association (fig.2). In contrast to these data for binding to whole cells, ^{125}I-EGF binding to acinar membrane preparations was not affected by the concentrations of Ca^{2+} in the medium in either the presence or absence of CCK_8 (not shown).

3.3. *Effects of Ca^{2+}-mediated secretagogues on EGF binding to A431 cells*

We also measured ^{125}I-EGF binding to cultures of A431 cells, a cell line which has an unusually high number of EGF receptors [11]. The effects of CCK_8, carbachol, and A23187 on ^{125}I-EGF binding to A431 cells is shown in table 1. CCK_8 and carbachol had no effect on EGF binding to these cells. In contrast, A23187 significantly inhibited

Table 1

Effects of secretagogues on ^{125}I-EGF binding to A431 cells

Condition	^{125}I-EGF bound % control
CCK_8 (10 nM)	95.2 ± 13.5 (3)
Carbachol (1 mM)	100.1 ± 15.0 (2)
A23187 (5 μM), without Ca^{2+}	104.7 ± 15.0 (2)
A23187 (5 μM), plus Ca^{2+} (1.25 mM)	60.2 ± 10.0 (6)

Cultures were incubated at 37°C for 1 h with 100 pM ^{125}I-EGF in the presence or absence of the various secretagogues. The Ca^{2+} concentration in the medium was 1.25 mM unless otherwise noted. Each value represents the mean ± SD of ^{125}I-EGF binding expressed as a % of control binding for the number of experiments given in parentheses

EGF binding by 40 ± 4% (n = 6) in the presence, but not the absence of Ca^{2+} in the binding medium. The absence of medium Ca^{2+} did not decrease the level of inhibition of EGF binding to acini induced by secretagogues (not shown). Since these secretagogues are able to increase intracellular free Ca^{2+} by causing release from intracellular pools, and this increase is sufficient to elicit most of the actions of these agents on acini [4], it is reasonable to assume that intracellular Ca^{2+} release also accounts for secretagogue-induced inhibition of EGF binding to acini in the absence of medium Ca^{2+}.

3.4. *Effect of EGF concentrations on inhibition of ^{125}I-EGF binding to pancreatic acini and A431 cells*

Competitive inhibition curves of ^{125}I-EGF binding to pancreatic acini were carried out with varying concentrations of unlabeled EGF in the presence and absence of CCK_8 (fig.3a). The inhibitory effects of CCK_8 were much greater at lower concentrations of ligand. Computer analyses of competitive inhibition curves indicated that the inhibition of ^{125}I-EGF binding by CCK_8 occurred with little change in maximal binding of EGF (30 ± 6% increase, n = 3) while the concentration of EGF required for half-maximal binding was more than doubled from 0.4 in the control to 1.0 nM in the presence of CCK_8 (135 ± 8% increase). Thus

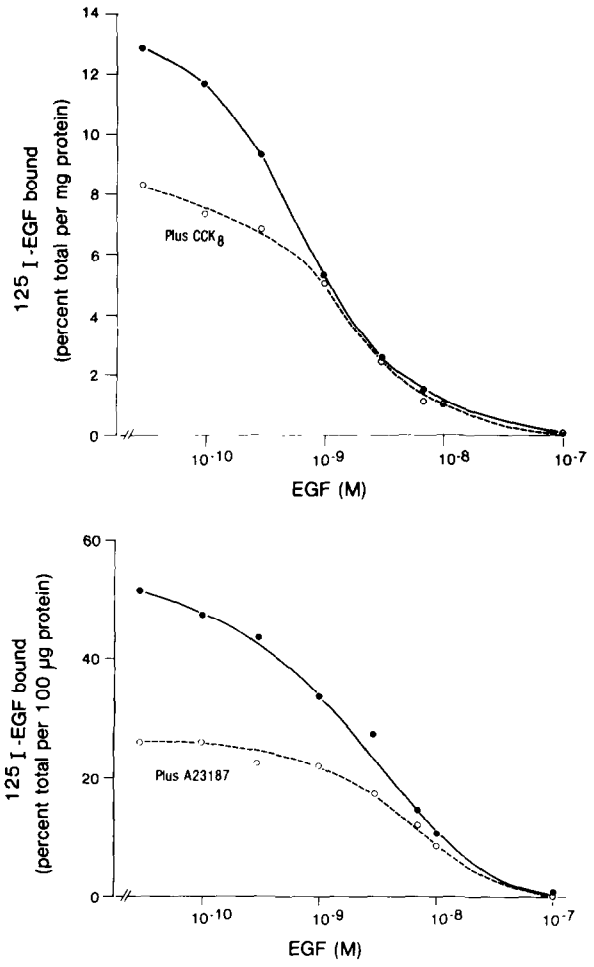

Fig.3. Competition-inhibition curves of ^{125}I-EGF binding to pancreatic acini and A431 cells. (a) Binding to acini in the presence and absence of CCK_8. (b) Binding to A431 cells in the presence and absence of A23187. Unlabeled EGF concentrations range from 0 to 100 nM; binding in the presence of 100 nM EGF was considered non-specific and subtracted from the other values. Incubations were carried out at 37°C for 2 h in the case of acini, or for 1 h in the case of A431 cells. Results shown are representative of 2–4 experiments.

CCK_8 leads to an apparent decrease in EGF receptor affinity.

A competition-inhibition curve for EGF binding to A431 cells in the presence or absence of A23187 plus Ca^{2+} is shown in fig.3b. The effects of A23187 were also greater at lower EGF concentrations and no change in maximal binding was noted. The concentration of non-radioactive EGF required for half-maximal inhibition of ^{125}I-EGF binding was doubled from 2.5 nM in the control to 5 nM in the presence of A23187 plus Ca^{2+}.

4. DISCUSSION

The experiments presented here show that CCK_8, desulfated CCK_8, carbachol and the ionophore A23187 inhibit the association of ^{125}I-EGF with pancreatic acini. Several lines of evidence suggest that this inhibition is not due to a direct competition for EGF receptors:

(i) These pancreatic secretagogues are chemically diverse, and are structurally unrelated to EGF;
(ii) Inhibition of ^{125}I-EGF binding is incomplete even at maximal doses of these agents;
(iii) CCK_8, the most potent inhibitor, had no effect on EGF binding to acinar membrane particles;
(iv) Neither CCK_8 nor carbachol inhibited ^{125}I-EGF binding to its receptor on A431 cells (which do not have CCK and muscarinic receptors).

It is highly probable that the inhibitory effects of these secretagogues are mediated by intracellular calcium. Support for this hypothesis comes from the following observations:

(i) Intracellular levels of Ca^{2+} in pancreatic acinar cells are increased by CCK_8, carbachol and A23187 [4,6,12];
(ii) All major effects of these secretagogues on pancreatic acinar cells are believed to be mediated by Ca^{2+} [4–6];
(iii) The relative potencies of each secretagogue on inhibition of ^{125}I-EGF binding is in parallel with its ability to cause Ca^{2+} efflux from acini [12,13];
(iv) the Ca^{2+} ionophore A23187 inhibited EGF binding to A431 cells in the presence but not in the absence of medium Ca^{2+}.

The observed inhibition of EGF binding occurred as an apparent affinity change. While this could indicate an effect of intracellular calcium on the affinity of the EGF receptor, association of EGF and other ligands with their target cells involves a complex series of events including binding, internalization and processing [14]. The inhibition of ^{125}I-EGF association with acini and A431 cells could therefore result from an effect of calcium on any of these events.

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health grant AM26422 and the Elise Stern Haas Research Fund of Mount Zion Hospital and Medical Center. C.L. was supported by a fellowship from the Cystic Fibrosis Foundation.

REFERENCES

[1] Logsdon, C.D. and Williams, J.A. (1983) Gastroenterology 85, 339–345.
[2] Korc, M., Matrisian, L.M., Planck, S.R. and Magun, B.E. (1983) Biochem. Biophys. Res. Commun. 111, 1066–1073.
[3] Logsdon, C.D. and Williams, J.A. (1983) Am. J. Physiol. 244, G675–G682.
[4] Williams, J.A. (1980) Am. J. Physiol. 238, G269–G279.
[5] Schulz, I. (1980) Am. J. Physiol. 239, G335–G347.
[6] O'Doherty, J. and Stark, R.J. (1982) Am. J. Physiol. 242, 6513–6521.
[7] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[8] Sankaran, H., Goldfine, I.D., Bailey, A., Licko, V. and Williams, J.A. (1982) Am. J. Physiol. 242, G250–G257.
[9] Peikin, S.R., Costenbader, C.L. and Gardner, J.D. (1979) J. Biol. Chem. 254, 5321–5327.
[10] Sakamoto, C., Williams, J.A., Wong, K.Y. and Goldfine, I.D. (1983) FEBS Lett. 151, 63–66.
[11] Fabricant, R.N., DeLarco, J.E. and Todaro, G.J. (1977) Proc. Natl. Acad. Sci. USA 74, 565–569.
[12] Gardner, J.D., Conlon, T.P., Klaeveman, H.L., Adams, T.D. and Ondetti, M.A. (1975) J. Clin. Invest. 56, 366–375.
[13] Christophe, J.P., Frandsen, E.K., Conlon, T.P., Krishna, C. and Gardner, J.D. (1976) J. Biol. Chem. 251, 4640–4645.
[14] King, A.C. and Cuatrecasas, P.L. (1981) New England J. Med. 305, 77–88.