Intracellular Ca^{2+} and phorbol esters synergistically inhibit internalization of epidermal growth factor in pancreatic acini

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The association of ^{125}I -labelled epidermal growth factor (^{125}I -EGF) with mouse pancreatic acinar cells was inhibited by secretagogues which increase intracellular free Ca^{2+} concentrations. These agents included cholecystokinin-octapeptide (CCK_8) and the Ca^{2+} ionophore A23187. Inhibition by CCK_8 was blocked by lowering the incubation temperature from 37°C to 15°C . Moreover, in contrast with studies of intact acini, the binding of ^{125}I -EGF to isolated acinar membrane particles was not affected either by CCK_8 , or by varying the level of Ca^{2+} in the incubation medium. These results indicated, therefore, that the inhibition of ^{125}I -EGF association with acinar cells required intact cells that are metabolically active. Since intact cells at 37°C are known to internalize bound EGF rapidly, acid washing was used to distinguish membrane-associated hormone from internalized hormone. Under steady-state conditions 86% of the ^{125}I -EGF associated with the acini was found to be internalized by this technique. When agents that increased intracellular Ca^{2+} were tested they all markedly reduced the amount of internalized hormone, whereas surface binding was only minimally affected. The phorbol ester 12-*O*-tetradecanoylphorbol 13-acetate (TPA), which is known to activate protein kinase C, a Ca^{2+} -regulated enzyme, also inhibited the association of EGF with acini. This inhibition was similar to that induced by elevated intracellular Ca^{2+} . To test whether these two inhibitory phenomena were related, the effects of TPA in combination with the Ca^{2+} ionophore A23187 were examined. At low concentrations the effects were synergistic, whereas at high concentrations the maximal level of inhibition was not changed. We suggest therefore that elevated intracellular Ca^{2+} and phorbol esters may inhibit EGF internalization by a mechanism involving activation of protein kinase C.

EGF is a 6100Da polypeptide that binds to cell surface receptors and induces numerous biological effects in a variety of cells (Carpenter & Cohen, 1979). The binding of EGF to its receptor on several cell types has been shown to be altered rapidly by structurally unrelated tumour- and growth-promoting agents. Certain agents, such as the polypeptides produced by fibroblasts transformed by murine sarcoma virus, bind directly to EGF receptors and decrease the number of receptors available for EGF binding on the plasma membrane (Shoyab *et al.*, 1979; De Larco &

Todaro, 1980; Ozanne *et al.*, 1980). In contrast, the inhibitory effects of other agents, including phorbol esters (Lee & Weinstein, 1978, 1979; Shoyab *et al.*, 1979; Brown *et al.*, 1979; Murray & Froscio, 1980; Magun *et al.*, 1980; Saloman, 1981), vasopressin (Rozengurt *et al.*, 1981) and fibroblast-derived growth factor (Rozengurt *et al.*, 1982) are indirect. These agents do not bind to the EGF receptor but rather interact with their own receptors and induce an apparent decrease in the EGF receptor affinity. The mechanism of action of these latter agents is however unknown.

It has been reported that pancreatic acini possess EGF receptors (Logsdon & Williams, 1983a; Korc *et al.*, 1983a) and that EGF promotes the maintenance of differentiated acinar cell

Abbreviations used: EGF, epidermal growth factor; CCK_8 , cholecystokinin-octapeptide; TPA, 12-*O*-tetradecanoylphorbol acetate.

function in culture (Logsdon & Williams, 1983a,b). We and others have also reported that cholecystokinin (CCK), a polypeptide hormone structurally unrelated to EGF, inhibited the uptake of ^{125}I -EGF by these cells and that this inhibitory effect was shared by several other agents whose actions in acini are mediated by Ca^{2+} (e.g. carbachol, desulphated CCK) (Logsdon & Williams, 1983c; Korc *et al.*, 1983b, 1984).

In the present study we further investigated this inhibitory effect of increased intracellular Ca^{2+} and compared it with the inhibitory effects of phorbol esters. We found that both increased intracellular Ca^{2+} and phorbol esters inhibit the association of ^{125}I -EGF to cells, primarily by blocking hormone internalization. We also found that phorbol esters and increased intracellular Ca^{2+} interacted in a synergistic manner, suggesting a common mechanism of action.

Experimental

Materials

Murine EGF (receptor grade) was purchased from Collaborative Research Inc., Waltham, MA, U.S.A.; chromatographically purified collagenase from Worthington Biochemicals, Freehold, NJ, U.S.A.; soya bean trypsin inhibitor type 1-S and 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (Hepes) from Sigma Chemical Co.; A23187 from Calbiochem-Behring, La Jolla, CA, U.S.A.; minimal Eagle's medium amino acid supplement and Waymouths 752/1 from GIBCO, Grand Island, NY, U.S.A.; bovine serum albumin, fraction V, from Miles Laboratories, Elkhart, IN, U.S.A., and Reheis, Chicago, IL, U.S.A.; carrier-free Na^{125}I from New England Nuclear; foetal bovine serum from Sterile Systems Inc., Logan, UT, U.S.A. CCK₈ was a gift from Dr. Miguel Ondetti, Squibb Institute for Medical Research, Princeton, NJ, U.S.A.

Cell culture

Isolated mouse pancreatic acini from fed adult male Swiss Webster mice were prepared and cultured as previously described (Logsdon & Williams, 1983b). Briefly this procedure involved injection of Krebs-Henseleit bicarbonate (KHB) buffer containing collagenase into the interstitium of the pancreatic tissue at 37°C for 50 min with shaking, then mechanical disruption by suction of the tissue through pipette orifices of decreasing diameter and centrifugation through gradients of KHB buffer containing 4% (w/v) bovine serum albumin. Acini were then washed and incubated as suspension cultures in Waymouths medium containing 20 mM-Hepes buffer (pH 7.4), 100 units of penicillin/ml, 100 µg of streptomycin/ml, 50 µg of

amphotericin/ml, 0.2 mg of soya bean trypsin inhibitor/ml, and 20% (w/v) foetal bovine serum at 37°C in a humidified atmosphere of CO_2/air (1:19). All experiments on acini were conducted after 24 h of culture.

Preparation of membrane particles

Acinar cell membrane particles were prepared as described previously (Steigerwalt & Williams, 1981). Acini, cultured for 24 h as described above, were washed twice and resuspended in 5 ml of 50 mM-Tris/HCl buffer (pH 7.4) containing 0.2 mg of soya bean trypsin inhibitor/ml at 4°C. The acini were homogenized by 15 up-and-down passes of a motor-driven Teflon/glass homogenizer. The homogenate was then centrifuged at 38000g for 15 min. The pellet was resuspended in the aforementioned Tris buffer and recentrifuged at 38000g for 15 min. Protein content was determined by the method of Bradford (1976).

Binding studies

EGF was iodinated by a chloramine-T method (Logsdon & Williams, 1983a). The eluted iodinated peptide was 92–98% precipitable by 10% (w/v) trichloroacetic acid and had a specific radioactivity of 100–130 Ci/g.

For measurements of peptide binding, acini (0.1–0.2 mg of acinar protein/ml) were washed free of culture medium and resuspended in a Hepes-buffered Ringer (HR buffer) containing 130 mM-NaCl, 4.7 mM-KCl, 1.0 mM- Na_2HPO_4 , 1.13 mM- MgCl_2 , 1.28 mM- CaCl_2 , 5.6 mM-glucose, and 10 mM-Hepes (pH 7.40) which was enriched with minimal Eagle's medium amino acid supplement, 0.1 mg of soya bean trypsin inhibitor/ml, 5 mg of bovine serum albumin/ml, and gassed with 100% O_2 . ^{125}I -EGF (40–60 pM) plus various concentrations of unlabelled hormones and secretagogues were added to the suspension and incubated in a shaking water bath (60 cycles/min) at 37°C. At specified times, triplicate 1 ml samples of the suspension were removed, centrifuged at 300g for 2 min at 4°C, and the pellets then washed twice with 0.15 M-NaCl at 4°C. Radioactivity associated with the washed acinar pellet was measured in a γ -radiation scintillation counter, and the acinar protein was measured. Duplicate aliquots of the incubation mixture were taken at the end of the incubation period and used to compute the percentage bound ^{125}I -EGF. Non-specific binding was determined by incubating acini with labelled EGF in the presence of an excess of unlabelled EGF (100 nM).

To measure the dissociation of bound ^{125}I -EGF, acini were incubated with ^{125}I -EGF (50 pM) for 2 h at 37°C. Acini were then rapidly resuspended in fresh HR buffer with or without 10 nM-CCK₈ at

37°C. Samples were taken at the indicated time points and bound ligand determined as above. For binding to membrane particles, the particles (0.2mg of protein/ml) were suspended in 10ml of HR buffer at 37°C in a shaking water bath at 60 cycles/min in the presence of 50pM-¹²⁵I-EGF. At each time point triplicate 300μl aliquots were layered over 100μl of ice-cold HR buffer in 400μl plastic microcentrifuge tubes. These were centrifuged at 10000g for 1 min in a microcentrifuge at room temperature. The supernatant was aspirated, and the pellet was washed with 200μl of ice-cold HR buffer. Samples were then recentrifuged for 15s. The supernatant was aspirated, the tip of the tube containing the pellet was cut off, and the radioactivity in the tip was determined in a γ-radiation scintillation counter.

Hormone internalization

The internalization of hormone was estimated by use of the acid wash procedure first described by Haigler *et al.* (1980). Samples were taken as described above, after which the acinar pellet was resuspended in 1 ml of 0.2M-acetic acid with 0.5M-NaCl (pH 2.5) for 5 min at 4°C. The cells were then recentrifuged at 300g for 2min at 4°C and the supernatant removed and counted for acid-removable, i.e. surface-bound, radioactivity. The pellet was washed once more in 0.15M-NaCl at 4°C and counted separately for acid-resistant, i.e. internalized, radioactivity.

Results

Time course and reversibility of CCK₈ induced inhibition of ¹²⁵I-EGF association

We previously reported that increasing the intracellular Ca²⁺ concentration in both pancreatic acinar and A431 cells decreased ¹²⁵I-EGF association (Logsdon & Williams, 1983c). Competition experiments indicated that this decreased association was due to a decrease in apparent receptor affinity. To elucidate further the mechanism of this inhibition we tested its time dependence and reversibility. The time course of ¹²⁵I-EGF association to pancreatic acini at 37°C in the presence and absence of CCK₈ is shown in Fig. 1. In the absence of CCK₈, maximal steady-state cell-associated radioactivity was achieved at 90min, and a plateau was maintained up to 210min. CCK₈ added simultaneously with EGF caused a marked reduction in cell-associated radioactivity which was evident within 15 min and persisted for at least 3.5 h (Fig. 1). CCK₈ was also able to decrease significantly EGF binding when added after equilibrium levels had been reached, although only partial reduction was obtained after

Fig. 1. Time-course of CCK₈-induced inhibition of specific ¹²⁵I-EGF binding to acini and its reversibility by dibutyryl cyclic GMP

Binding was conducted at 37°C in four flasks; at time zero two received 50pM-¹²⁵I-EGF tracer (○, ●) and two received tracer plus 10nM-CCK₈ (□, ■). After 90 min, 10nM-CCK₈ was added to one tracer-only flask (○) and 1mM-dibutyryl cyclic GMP (Bt₂cGMP) to one of the CCK₈ plus tracer flasks (□). Samples were taken at the indicated time intervals and cell-associated radioactivity was determined. Results are means ± s.d. of triplicate determinations from a representative of six experiments.

2h. The addition of dibutyryl cyclic GMP, a competitive antagonist of CCK binding, after 90 min of incubation with ¹²⁵I-EGF in the presence of CCK₈ led to reversal of the inhibitory effect. However, this reversal was slow and incomplete. In contrast with its effects on association, the presence of CCK₈ caused no change in the rate of dissociation of prebound ¹²⁵I-EGF (Fig. 2). These data indicated therefore, that CCK₈ affected the association of EGF with acini interfering with a dynamic process which continued during steady-state conditions, and that once initiated, the inhibitory effect was slow to reverse.

Effect of temperature on inhibition by CCK₈ of ¹²⁵I-EGF binding to pancreatic acini

The effect of reduced temperature on the association of ¹²⁵I-EGF with pancreatic acini and on the ability of CCK₈ to inhibit this association is shown in Fig. 3. The rate of association of ¹²⁵I-EGF with the cells at 15°C was much slower than at 37°C; the amount of cell-associated radioactivity was low and still increasing even after 8 h. When compared with 37°C, at 15°C CCK₈ added simultaneously with ¹²⁵I-EGF to acini had a reduced effect. In similar experiments carried out at 4°C, association was extremely low and no inhibition of ¹²⁵I-EGF binding by CCK₈ was

Fig. 2. Effect of CCK₈ on dissociation of ¹²⁵I-EGF from acini

Acini were incubated at 37°C for 2 h in the presence of 100 pM-¹²⁵I-EGF, washed free of unbound EGF and resuspended in buffer with (○) or without (●) 10 nM-CCK₈ for the indicated time. Results are means ± S.D. for triplicate determinations from a representative of three experiments.

noted (results not shown). ¹²⁵I-EGF association was inhibited by excess non-radioactive EGF at either temperature. These results support the hypothesis that CCK₈ does not compete directly for EGF receptors and suggest that the inhibitory effect of CCK₈ involves a temperature-dependent step. To investigate further the effects of temperature on the CCK₈-induced inhibition, acini were preincubated with CCK for 30 min at 37°C before the temperature was reduced to 15°C and the binding of ¹²⁵I-EGF was initiated (Fig. 3). Under these conditions the association of ¹²⁵I-EGF was significantly inhibited, but not to as great an extent as when the entire experiment was carried out at 37°C.

Lack of effect of CCK₈ on EGF binding to pancreatic membrane particles

Since the CCK₈-induced inhibition of EGF uptake is not a direct competition by CCK for EGF receptors, but required a temperature-dependent step, we tested whether the effect would also occur in isolated membrane fractions. Accordingly, pancreatic membrane particles were prepared which specifically bound ¹²⁵I-EGF (Fig. 4). In contrast with intact cells, CCK₈ did not inhibit the binding of ¹²⁵I-EGF to isolated acinar membranes. Moreover, membranes prepared from acini which had been pretreated with CCK₈ showed no difference in binding properties compared with those from untreated cells (results not shown). These results were consistent with an indirect mechanism of CCK₈ to inhibit EGF

Fig. 3. Effect of temperature on the CCK₈-elicited inhibition of EGF binding

Acini were preincubated for 30 min at 37°C then transferred to a 15°C shaking waterbath for an additional 10 min before 50 pM-¹²⁵I-EGF was added and the binding time-course carried out. Four flasks were used; one received only tracer ¹²⁵I-EGF (●), one had 10 nM-CCK₈ included during the preincubation (□), one received 10 nM-CCK₈ simultaneously with the tracer (■) and one received an excess of non-radioactive EGF (○). Results are the means ± S.E.M. for three experiments.

Fig. 4. Time-course of ¹²⁵I-EGF binding to pancreatic membrane particles

Acinar cell membrane particles (0.2 mg of protein/ml) were incubated at 37°C with 50 pM-¹²⁵I-EGF in the presence (○, □) or absence (●, ■) of 10 nM-CCK₈. Non-specific binding (■, □) was defined as that detected in the presence of 100 nM-unlabelled EGF. Results are means ± S.E.M. for three experiments.

binding. They also raised the possibility that some cytoplasmic process was involved in the inhibition.

Effect of increased intracellular Ca²⁺ on the internalization of ¹²⁵I-EGF

Since the association of ¹²⁵I-EGF with acini occurred very slowly at reduced temperatures (see Fig. 3), subsequent experiments were conducted at 37°C. In order to assess the magnitude of EGF internalization at this temperature in acini (and the effects of CCK₈ on this process), we used an acid-washing technique, a procedure which dissociates hormone which is bound to receptors located at the cell surface (Haigler *et al.*, 1980). At 37°C, 86% of the hormone associated with the cell at steady state was in an acid-resistant space, i.e. internalized (Fig. 5). (The terms acid-resistant and acid-removable are more accurate, since this technique may not give 100% resolution. In two control experiments where binding was conducted for 4 h at 4°C, a temperature at which internalization should be halted, acid-washing indicated that 23% was acid-resistant. Therefore it seems likely that a portion of the 'internalized' hormone is acid-resistant but may not actually be localized within the cell.) The major effect of CCK₈ was to reduce this component of EGF association while having a much smaller effect on the acid-removable, i.e. surface-bound, ligand.

Fig. 5. Time-course of ¹²⁵I-EGF internalization by acini. Acini were incubated at 37°C with 50 pM ¹²⁵I-EGF tracer (●, ■) or tracer plus 10 nM CCK₈ (○, □). Samples were taken at the indicated time intervals and subjected to acid-washing. Specific acid-resistant, i.e. internalized, radioactivity (○, ●) and radioactivity removed by the acid treatment, i.e. surface-bound (□, ■) were determined. Results are means ± S.E.M. of three experiments.

Effects of phorbol esters on EGF binding to pancreatic acini

The inhibitory effects on EGF binding of increased intracellular Ca²⁺ shares many characteristics with the inhibitory effects of phorbol esters reported by others in a number of cell types (Lee & Weinstein, 1978, 1979; Shoyab *et al.*, 1979; Brown *et al.*, 1979; Murray & Froscia, 1980; Magun *et al.*, 1980; Saloman, 1981). In each case inhibition is indirect, temperature-dependent and not associated with a change in hormone degradation. Although there have been reports that phorbol esters decrease the number of available EGF receptors per cell (Lee & Weinstein, 1978, 1979; Murray & Froscio, 1980; Magun *et al.*, 1980; Saloman, 1981) changes in apparent affinity of the EGF receptor have also been reported (Shoyab *et al.*, 1979; Brown *et al.*, 1979; Magun *et al.*, 1980) as was seen in pancreatic acini with increased intracellular Ca²⁺ (Logsdon & Williams, 1983c). To determine whether phorbol esters might be having an effect on hormone internalization similar to that caused by increased intracellular Ca²⁺, we again used the technique of acid washing. Fig. 6 shows that TPA inhibits the association of ¹²⁵I-EGF with acini in a concentration-dependent manner. As there is a dramatic decrease in the amount of acid-resistant radioactivity (i.e. internalized) and little change in the amount of acid-removable radioactivity (i.e. surface-bound) associated with the acini after TPA treatment we conclude that the major effect of

Fig. 6. Dose-response of TPA on ¹²⁵I-EGF association with acini

Acini were incubated at 37°C with 50 pM ¹²⁵I-EGF and the indicated concentrations of TPA for 2 h before samples were taken and subjected to acid-washing. Specific acid-resistant, i.e. internalized, radioactivity (●) and radioactivity removed by the acid treatment, i.e. surface-bound (○), were determined. Results are means ± S.E.M. of six experiments.

Fig. 7. Interaction of TPA and the Ca^{2+} -ionophore A23187 on ^{125}I -EGF internalization by acini

Acini were incubated at 27°C with 50 pM - ^{125}I -EGF for 2 h in the presence of various concentrations of TPA alone (●) or TPA plus $1\text{ }\mu\text{M}$ -A23187 (○). The percent maximal specific acid-resistant, i.e. internalized, radioactivity is shown. Results are the means $\pm$ S.E.M. of three experiments.

TPA is on hormone internalization. Significant inhibition was seen with 0.3 nM -TPA and maximal inhibition of H^+ -resistant radioactivity was seen at 10 – 30 nM . In similar experiments the relatively inactive phorbol derivative 4-B-phorbol used at a concentration of 10 nM led to a $25.5 \pm 11.9\%$ ($n = 3$) inhibition of specific H^+ -resistant radioactivity as compared with $83.6 \pm 1.9\%$ ($n = 3$) in the same experiments for a similar concentration of TPA. Since 4-B-phorbol was relatively inactive, the response to phorbol derivatives by acini correlates with reported structure-activity relationships for tumour promotion (Weinstein *et al.*, 1982).

Interaction of increased intracellular Ca^{2+} and TPA on EGF internalization

Since both TPA and elevated intracellular Ca^{2+} had similar effects on the association of EGF with acini, we tested them in combination. Fig. 7 shows the effects of $1\text{ }\mu\text{M}$ -A23187 in the presence of various concentrations of TPA on the internalization of ^{125}I -EGF. This concentration of A23187 itself had no effect on EGF association. However, in the presence of 10 pM -TPA, a concentration which also alone had no effect, there was a synergistic inhibition of ^{125}I -EGF internalization. This interaction was greatest at low TPA concentrations, whereas there was little change in the maximal effect.

Discussion

We have previously reported that increased intracellular Ca^{2+} inhibited the association of EGF with pancreatic acinar and A431 cells

(Logsdon & Williams, 1983c). This conclusion was based on the observation that several pancreatic secretagogues could each induce a decrease in cell-associated EGF in proportion to their known abilities to stimulate secretion (Logsdon & Williams, 1983c; Korc *et al.*, 1983b, 1984). Numerous studies on the roles of these agents as secretagogues for pancreatic enzyme release have led to acceptance of the idea that their secretory effects are mediated by increased intracellular Ca^{2+} concentration (for review see Williams, 1980). Moreover, recent measurements made with the fluorescent probe quin-2 have directly confirmed that the interaction of two of these agents, CCK and carbachol, with their receptors on pancreatic acinar cells leads to an increase in intracellular levels of free Ca^{2+} (Ochs *et al.*, 1983). On the other hand, these secretagogues have also been shown to activate the breakdown of inositol lipids (Laychock & Putney, 1982), leading to the formation of diacylglycerol. Diacylglycerol itself may be an important cellular activator, and as yet there is no clear consensus as to the relative importance of these two mediators.

In the present study we report that phorbol esters, like increases in intracellular Ca^{2+} , inhibited the association of EGF with pancreatic acini, and that there is a synergistic interaction between phorbols and intracellular Ca^{2+} . Although phorbol esters do not increase Ca^{2+} efflux from pancreatic acini, they mimic the actions of increased intracellular Ca^{2+} on these cells (Gunther, 1981). Froscio *et al.* (1981) have reported that the inhibitory effects of phorbol esters on EGF binding are blocked by agents which inhibit the interaction between Ca^{2+} and Ca^{2+} -dependent regulatory proteins. These and other similarities between the actions of phorbol esters and Ca^{2+} can be explained by the recent finding that the phorbol esters binding protein co-purifies with the phospholipid-dependent Ca^{2+} -activated protein kinase C (Niedel *et al.*, 1983). Castagna *et al.* (1982) have shown that phorbol esters interact directly with this kinase, act as substitutes for diacylglycerol, and thereby greatly increase the affinity of the kinase for Ca^{2+} . Synergistic activation of protein kinase C has been suggested as an explanation for the effectiveness on stimulation of platelet secretion of combinations of Ca^{2+} ionophore and TPA at concentrations which alone were ineffective (Rink *et al.*, 1983). This could also explain the synergistic interactions of TPA and increases in intracellular Ca^{2+} on EGF internalization reported here. The presence of protein kinase C has been detected in pancreatic acinar cells (Burnham & Williams, 1984). Since the interaction between several pancreatic secretagogues and their receptors on pancreatic acinar cells leads

to the breakdown of inositol lipids, yielding diacylglycerol (Laychock & Putney, 1982), and diacylglycerol can directly activate protein kinase C (Castagna *et al.*, 1982), this may be a physiologically important regulatory event, along with increases in intracellular Ca²⁺.

In the previous study, competitive binding experiments carried out in the presence and absence of maximally inhibitory concentrations of CCK suggested an apparent change in receptor affinity (Logsdon & Williams, 1983c). However, in the present study we found that there were high levels of ligand internalization at equilibrium, which nullify the assumptions necessary for interpretation of equilibrium binding data. Moreover, in the present report the data strongly indicate that the primary effect of increased intracellular Ca²⁺ concentrations was an inhibition of hormone internalization. First, inhibition was abolished at low temperatures where internalization is slow or absent. Second, binding of ¹²⁵I-EGF to isolated membrane particles was not affected by the secretagogues or medium Ca²⁺. Finally, inhibition was associated with a dramatic decrease in cellular uptake of ligand into an acid-resistant compartment, whereas there was little change in the level of acid-removable ligand. Similar results have been reported by Korc *et al.* (1984). Thus, although most reports of inhibitory effects of various ligands on EGF association with cells have suggested an apparent affinity change (Shoyab *et al.*, 1979; Brown *et al.*, 1979; Magun *et al.*, 1980; Rozengurt *et al.*, 1981, 1982), our data suggest that an effect on ligand internalization should also be considered. Furthermore, inhibitory effects of phorbol esters on EGF binding have also been previously reported (Lee & Weinstein, 1978; Shoyab *et al.*, 1979; Brown *et al.*, 1979; Murray & Froscio, 1980; Lee & Weinstein, 1979; Magun *et al.*, 1980; Saloman, 1981); however, there has been little agreement on the nature of this inhibition. Changes in EGF internalization induced by phorbol esters might explain some of the discrepancies.

Inhibition of EGF association by intracellular Ca²⁺, or products of inositol lipid breakdown, may also explain the inhibitory effects on EGF association seen in other cell types induced by various other ligands (e.g. Rozengurt *et al.*, 1981, 1982). For instance, vasopressin, which decreases the association of EGF with 3T3 cells (Rozengurt *et al.*, 1981), is known to induce other biological actions via effects on intracellular Ca²⁺ (Hems & Whittan, 1980).

There are several possible substrates whose phosphorylation might affect hormone internalization. It has recently been shown that phorbol esters interact with protein kinase C to induce a

change in the phosphorylation of the EGF receptor in A431 cells (Iwashita & Fox, 1984; Cochet *et al.*, 1984). Other possible substrates for this phosphorylation might be coated vesicle or cytoskeletal components involved in receptor internalization (Naka *et al.*, 1983; Pfeffer *et al.*, 1983).

The physiological significance of these findings remains to be elucidated. Receptor-mediated internalization of ligands is a generalized phenomena occurring in many cell types and for many ligands (King & Cuatrecasas, 1981). The biological role for internalization is not completely understood, but may be involved in delivery of the ligand to intracellular sites of hormone action (Goldfine *et al.*, 1977), the production of an intracellular second messenger by proteolytic processing of internalized hormone or receptor (Fox & Das, 1979) or for hormone and/or receptor degradation (Savion *et al.*, 1980). In the present study, inhibition of EGF binding occurred within a physiological concentration range of CCK₈. One possibility, therefore, is that since both CCK (Johnson, 1981) and EGF (Dembinski *et al.*, 1982) each have trophic effects on the exocrine pancreas, the ability of CCK to modulate EGF receptor internalization may allow the two hormones to act in a concerted fashion.

In summary, the results of this study indicate that increased intracellular Ca²⁺ and phorbol esters synergistically inhibit EGF internalization by pancreatic acinar cells. We suggest that this is likely to be mediated by an interaction with the Ca²⁺-regulated protein kinase C. Similarities between the effects reported here and those of several tumour- and growth-promoting agents suggests the existence of a common mechanism. The finding that several unrelated ligands can alter the cellular internalization of another peptide by acting through a Ca²⁺ regulated mechanism may have widespread significance.

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