Vol. 94, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1980 Pages 630-637

# INHIBITORS OF $^{125}$I-EPIDERMAL GROWTH FACTOR INTERNALIZATION

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Received April 14,1980

## SUMMARY

The binding and internalization of $^{125}$I-EGF by cultured rat cells was studied using a biochemical method that allows a clear discrimination between cell-surface bound and internalized hormone. In control cultures $^{125}$I-EGF initially bound to its receptor on the plasma membrane and then rapidly was internalized; one-half of the cell-bound hormone was internalized within 5 min at 37°. Bacitracin, methylamine, and diazooxonorvaline, compounds that inhibit both transglutaminase activity and $\alpha_2$-macroglobulin internalization, did not effect the rate at which $^{125}$I-EGF was internalized. Thus, although these two ligands are sequestered into the same endocytic vesicles, the molecular mechanism of clustering and internalization are different.

The internalization of $^{125}$I-EGF was inhibited by quinacrine, chlorpromazine, p-bromophenacylbromide, and phenylglyoxal. Possible mechanisms of action of these compounds are discussed.

## INTRODUCTION

The interaction between the polypeptide mitogen epidermal growth factor (EGF) and its cellular receptor has been extensively studied using radioactive (1,2), fluorescent (3,4), and ferritin (5) derivatives. Following binding to its plasma membrane receptor the EGF:receptor complex clusters in clathrin-coated pits, is internalized into endocytic vesicles and eventually is degraded. Fluorescent derivatives of EGF, insulin, and $\alpha_2$-macroglobulin ($\alpha_2$M) are sequestered in the same endocytic vesicles (6). Although considerable information has accumulated concerning the internalization pathway, very little is known above the molecular mechanism involved in clustering and internalization, or the sites(s) in this pathway at which EGF exerts its many biological effects. One approach to this problem is to identify compounds that inhibit the process of interest and then determine the mechanism by which the inhibitors exerts their effect. It has been reported that the ability of a diverse group of compounds to inhibit rhodamine-labeled $\alpha_2$-M clustering and internalization correlates well with their ability to inhibit the enzyme transglutaminase (7,8).

Recently we developed a biochemical method for measuring $^{125}$I-EGF binding and internalization that involves removal of cell-surface hormone by treatment

Abbreviations: DONV, 5-diazo-4-oxo-norvaline; EGF, epidermal growth factor; $\alpha_2$M, $\alpha_2$-macroglobulin; PLase $A_2$, phospholipase $A_2$.

0006-291X/80/100630-08$01.00/0

with acetic acid under conditions that do not remove internalized hormone (9). In this communication we use this method to show that the transglutaminase inhibitors methylamine and bacitracin are without effect on the internalization of $^{125}$I-EGF.

The enzyme phospholipase $A_2$ (PLase $A_2$) has been implicated in the response of cells to peptide chemoattractants (10), prolactin (11), corticotropin (12), platelet-derived growth factor (13), and thyrotropin (14). In this communication we show that three compounds that have been reported to inhibit PLase $A_2$ activity are inhibitors of $^{125}$I-EGF internalization.

## MATERIALS AND METHODS

Mouse EGF was isolated by the method Savage and Cohen (15) and was iodinated ($2.57 \times 10^5$ cpm/ng) by published procedures (1). Phenylglyoxal was obtained from Aldrich (Milwaukee, Wis.). Bacitracin, quinacrine, and p-bromophenacylbromide were purchased from Sigma Chemical Co. (St. Louis, Mo.). Monodansylcadaverine was obtained from Fulka (Switzerland).

An epithelioid clone of the normal rat kidney cell line, NRK-VB4, (16) was grown in Dulbecco-Vogt's modified Eagle's medium containing 10% (V/V) calf serum. Experimental cultures were plated in 35mm Falcon tissue culture dishes at a density of $1.0 \times 10^5$ cells/dish in 2 ml of medium supplemented with 10% calf serum heated to 60°C for 1 hr. Cultures were used 3 days after plating when confluent monolayers had formed; each dish contained approximately $1.9 \times 10^6$ cells. The number of cells per replicate dish varied by less than 10%. Cultures were washed twice with warm medium. Binding studies were then performed on duplicate dishes in 1 ml of Dulbecco-Vogt's medium (pH 7.4) at 37° containing 20 mM Hepes and 0.1% bovine serum albumin. After incubation with $^{125}$I-EGF the cultures were washed five times with binding media at 4°. The location of cell-bound hormone was determined as previously described (9). In brief, the cultures were treated at 4° for 6 min with acetic acid (0.2M, pH2.5 containing 0.5 M NaCl) to remove cell-surface bound hormone. The remaining internalized $^{125}$I-EGF was removed by solubilizing in 1N NaOH. Non-specific binding as determined by measuring the binding in an excess of unlabeled EGF (3 µg/ml) was approximately 5% of the total binding. All results are expressed as specific binding. Binding to duplicate dishes varied, in general, by less than 10%.

## RESULTS AND DISCUSSION

The effect of three compounds (quinacrine, (17) p-bromophenacylbromide, (18,19), and chlorpromazine (20) that have been reported to inhibit phospholipase $A_2$ (PLase $A_2$) activity were tested to determine if they altered the rate of $^{125}$I-EGF internalization. Quinacrine (800 µM) altered the total binding of $^{125}$I-EGF by less than 10%; however, the drug had a marked effect on internalization (Fig. 1). In control cultures maximal surface binding occured at 3.5 min and was followed by a decline in surface binding with an accumulation of internalized hormone. In quinacrine-treated cultures the amount of surface bound hormone did not begin to decrease until 7.5 min and the rate and extent of internalization was greatly reduced.

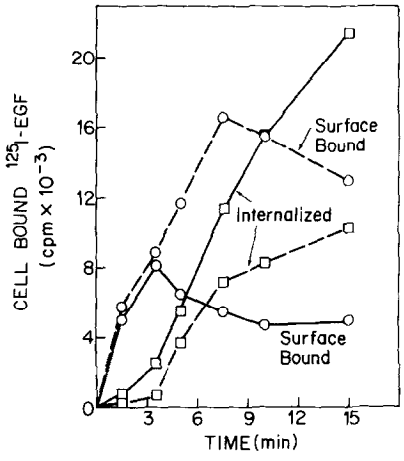


Fig. 1. Effect of quinacrine on the time course of $^{125}$I-EGF binding and internalization. Cultures of NRK cells were preincubated in the absence (solid lines) or presence (dashed lines) of quinacrine (800 μM) for 3 min at 37° then incubated with $^{125}$I-EGF (4ng/ml) for the indicated time at 37° in the same media. Cultures were washed at 4° and treated with acid to remove the cell-surface bound $^{125}$I-EGF (circles) and the cells were then lysed with NaOH to remove internalized hormone (squares).

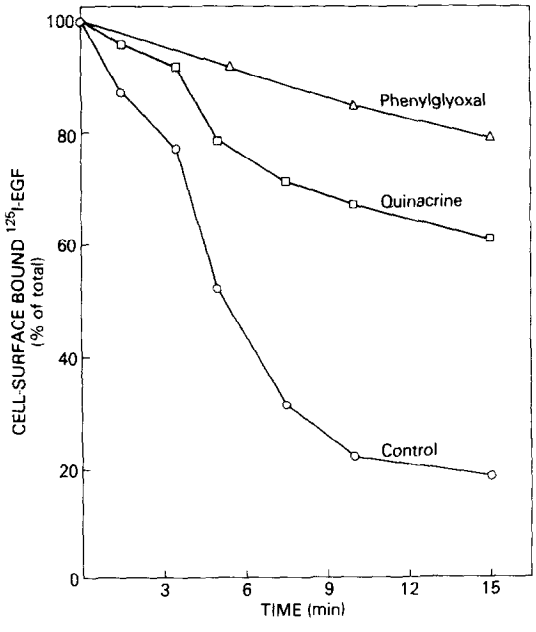


Fig. 2. Effect of phenglyglyoxal and quinacrine on $^{125}$I-EGF internalization. Cultures were pretreated at 37° with either phenylglyoxal (1.2 mM, 20 min triangles), quinacrine (800 μM, 3 min, squares) or control media (circles) and then incubated with $^{125}$I-EGF (4ng/ml) for the indicated time. After washing, the amount of $^{125}$I-EGF bound to the cell surface and interior was determined by treatment with acetic acid (see Materials and Methods). Quinacrine did not have a significant effect on the amount of total binding. Phenylglyoxal induced approximately a 20% increase in total binding.

The effect of quinacrine can be more clearly seen when the percentage of cell-bound $^{125}$I-EGF located inside the cell is plotted as a function of time (Fig. 2). In control cultures greater than 80% of the cell-bound hormone had been internalized within 15 min but less than 40% was internalized in quinacrine treated cultures after 15 min. The effect of quinacrine was reversible. Cultures that were pretreated with quinacrine (1.0 mM) for 10 min, washed to remove the inhibitor then incubated with $^{125}$I-EGF for 10 min, internalized the hormone at a rate very similar to control cultures. The concentration dependence of inhibition of internalization by quinacrine is shown in Fig. 3. Chlorpromazine (100-200 μM) and bromophenacylbromide (20 μM) were also efficient inhibitors of $^{125}$I-EGF internalization (Table I) but unlike quinacrine produced a 31-49% inhibition in total binding. Bromophenacylbromide produces an irreversible inhibition of PLase $A_2$ by modifying a histidine residue at the active site (18,19). Its effect on EGF internalization was not reversed by removal of the inhibitor from the medium.

Although these preliminary data suggest that a PLase $A_2$-like activity may be involved in EGF clustering and/or internalization additional experiments are required before a connection can be convincingly established. Experiments are under way to directly measure PLase $A_2$ activity in control and EGF stimulated cultures.

Since phenylglyoxal inhibits the internalization of $\alpha_2$-macroglobulin (21) and pinocytosis of surface bound lectins (22) in macrophages, the effect of

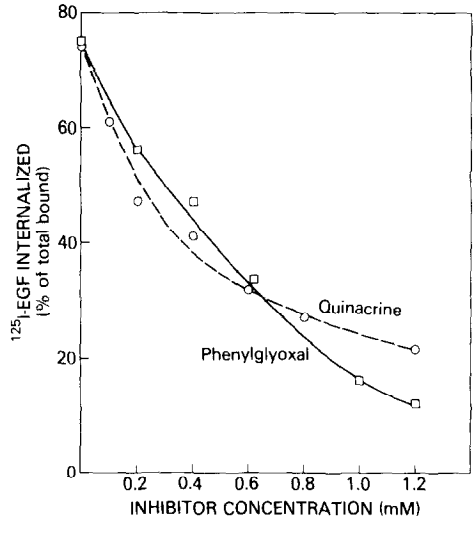


Fig. 3. Effect of quinacrine and phenylglyoxal concentration on $^{125}$I-EGF internalization. Cultures were preincubated with the indicated concentration of quinacrine (circles) or phenylglyoxal (squares) for 5 min or 20 min at 37°, respectively. $^{125}$I-EGF (2ng/ml) was added and incubated at 37° for 10 min. After washing, the amount of $^{125}$I-EGF bound to the cell surface and interior was determined by treatment with acetic acid (see Materials and Methods).

TABLE I

Cells were preincubated for 20 min at 37° with the inhibitor, then $^{125}$I-EGF (2 ng/ml) added for 10 min. DONV and p-bromophenacylbromide were added in DMSO such that the binding media contained 0.5% DMSO; 0.5% DMSO had no effect on hormone binding or internalization.

| Treatment | Concentration | Total binding (% of control) | $^{125}$I-EGF internalized (% of total bound) |
|---|---|---|---|
| control | -- | 100 | 70 |
| lidocaine | 3 mM | 119 | 66 |
| dansylcadaverine | 500 μM | 85 | 38 |
| methylamine | 25 mM | 121 | 70 |
| methylamine | 50 mM | 110 | 65 |
| DONV | 20 μM | 141 | 71 |
| DONV | 50 μM | 130 | 62 |
| bacitracin | 2 mg/ml | 107 | 71 |
| bacitracin | 6 mg/ml | 90 | 70 |
| chlorpromazine | 100 μM | 69 | 56 |
| chlorpromazine | 200 μM | 51 | 22 |
| bromophenacylbromide | 20 μM | 60 | 26 |
| quinacrine | 1 mM | 105 | 29 |

this compound on EGF internalization was investigated. Although phenylglyoxal (1.2 mM) had only minor effects on the total amount of $^{125}$I-EGF bound, Fig. 2 shows that the rate of $^{125}$I-EGF internalization was greatly reduced by phenylglyoxal. The inhibition was reversible; cells pretreated with phenylglyoxal (1.2 mM) for 30 min were capable of internalizing hormone at approximately control rates after the inhibitor was removed. The concentration dependence of inhibition of internalization by phenylygloxal is shown in Fig. 3. Phenylgloxyal does not enter cultured cells (23) and is a relatively specific reagent for modifying arginine side chains in proteins (24). The most straightforward interpretation of phenylglyoxal inhibition of EGF internalization is that the reagent is acting by modifying an arginine residue on a cell surface protein that is essential for clustering and/or internalization of the EGF receptor complex. However, this interpretation must be considered very preliminary until additional data are obtained.

It has been reported that a variety of compounds that inhibit the enzyme transglutaminase block the clustering and internalization of fluorescent derivatives of EGF and $\alpha_2$M (7,8). Several of these compounds were tested for their ability to inhibit $^{125}$I-EGF internalization. As previously reported for Balb cells (9) dansylcadaverine inhibits this process in NRK cultures (Table I) at concentrations that are high (500 μM) relative to the amount required to

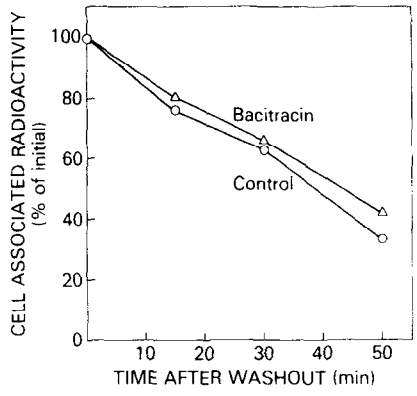


Fig. 4. Effect of bacitracin on the dissociation of cell-bound $^{125}$I-EGF. Cultures were preincubated at 37° for 5 min in the absence (circles) and presence (triangles) of bacitracin (2mg/ml). The cells were then incubated with $^{125}$I-EGF (3ng/ml) for 10 min at 37°. The cultures were washed five times with warm binding medium to remove unbound hormone and then incubated in hormone-free medium in the continued presence of inhibitor. At the indicated time the total cell bound radioactivity was determined. The results are expressed as the amount of cell-bound radioactivity at the indicated time relative to the amount cell-bound radioactivity at the end of the 10 minute incubation with $^{125}$I-EGF with or without bacitracin. Bacitracin did not significantly alter the amount of $^{125}$I-EGF that was initially bound.

inhibit R-$\alpha_2$M clustering (100 μM) (7). However, high concentrations of methylamine, bacitracin and the affinity label of transglutaminase, DONV (diazooxonorvaline), were without effect on internalization as assayed by the acetic acid release method (Table I). A number of compounds that inhibit cellular methyltransferase activity (25,26) were also without effect (data not shown).

The lack of effect of bacitracin on internalization was confirmed by the following experiment. Cultures were preincubated in the presence and absence of bacitracin (2 mg/ml) for 5 min. The cells were then incubated for 10 min with $^{125}$I-EGF, washed to remove unbound hormone, and then incubated at 37° in hormone-free medium in the continued presence of inhibitor. It has previously been shown that cell-bound $^{125}$I-EGF is internalized and degraded to $^{125}$I-tyrosine which is released into the medium (1). Fig. 4 shows that there is no significant difference in the rate of loss of radioactivity in control and bacitracin-treated cells. Furthermore, the radioactivity released into the medium was analyzed by Bio-gel P-10 chromatography (1) and 65% and 61% of the radioactivity comigrated with iodotyrosine in the control and treated cultures respectively. These results suggest that the hormone was internalized and degraded normally in the presence of bacitracin. In contrast phenylglyoxal-treated cultures lost the cell-bound radioactivity into the medium at a faster rate and 97% of the released radioactivity ran as intact $^{125}$I-EGF on a P-10

column (data not shown). This suggests that phenylglyoxal blocked internalization and the hormone dissociated from its cell-surface receptor intact.

The results reported herein concerning the lack of effect of bacitracin and methylamine on $^{125}$I-EGF internalization are not in agreement with previous results using a rhodamine-lactalbumin-EGF conjugate (7,8). To reexamine this point, we prepared a direct conjugate of rhodamine and EGF and found that bacitracin and methylamine did not block clustering and internalization of this direct conjugate (Haigler and Pastan, unpublished results). Thus, this fluorescent result is in agreement with the biochemical data reported herein. Previous morphological studies have shown that human fibroblasts internalize $^{125}$I-EGF in the presence of ammonium chloride (27) and that human carcinoma cells internalize ferritin conjugated EGF in the presence of primary amines (28). Thus, all information concerning amine inhibitors of internalization of EGF is in agreement except that obtained using a rhodamine-lactalbumin-EGF conjugate (7,8). The rhodamine-lactalbumin-EGF conjugate was prepared from a commercial preparation of EGF (Collaborative Research) which has since been shown to be a mixture of peptides. Therefore, these data (7,8) must be considered unreliable.

It has previously been shown that methylamine, bacitracin and DONV inhibit the normal uptake of $\alpha_2$-M (7,8, Levitzki and Pastan, unpublished results) and 3,3',5-triiodo-L-thyronine (29). Since $\alpha_2$-M, EGF, and 3,3',5-triiodo-L-thyronine are sequestered in the same endocytic vesicles (6,29), it is of interest that these compounds are without effect on EGF internalization. This suggests that although the anatomical pathway for internalization of these ligands is similar, the molecular mechanism(s) involved are different.

ACKNOWLEDGEMENTS

We would like to acknowledge the excellent technical assistance of Ms. E. Lovelace. H.T.H. was an American Cancer Society Postdoctoral Fellow.

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