EFFECT OF METHYLAMINE ON INTERNALIZATION, PROCESSING AND BIOLOGICAL ACTIVATION OF EPIDERMAL GROWTH FACTOR RECEPTOR

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1. Introduction

Epidermal growth factor (EGF), a mitogenic polypeptide of M_r 6045 interacts with responsive cells through high affinity surface receptors [1]. The membrane receptor for EGF is a polypeptide of M_r ~184 000 [2,3], and interaction of EGF with this surface receptor has been shown to lead to the intracellular generation of an activator of DNA replication [4]. As with many other polypeptide ligands, binding of EGF to the surface receptor has been shown to be followed by endocytic uptake of the hormone–receptor complex leading to subsequent proteolytic degradation of both EGF and receptor within lysosomes [3,5,6]. Our earlier studies had indicated that the phenomenon of EGF-induced receptor internalization may play an essential role in the induction of the message necessary for the mitogenic response [3,4]. We are therefore interested in using various inhibitory compounds which block one or more of the steps in the cascade of cellular reactions that follow hormone–receptor binding, and in studying their effect on the induction of the mitogenic message. There have been reports on the inhibitory effects of various amines on the biological fate of hormones bound to target cells [5,7,8]. Here we report the effect of methylamine (MA) on the interaction of EGF with murine 3T3 cells. We find that the crucial event in hormone–cell interaction which is blocked by MA is not the initial endocytic event, but rather the later intracellular proteolytic processing of hormone and receptor. We also report some of the effects of MA on EGF-induced stimulatory responses.

2. Materials and methods

2.1. *General procedures*

Monolayer cultures of Swiss mouse 3T3 cells were grown and maintained as in [4]. Pure murine EGF [9] was labeled with ^{125}I (Amersham) as in [2,3]. Methylamine was from Sigma Chemical Co. The procedure for binding of ^{125}I-EGF to cell monolayers has been described [2,3], and was used without further modification. Incorporation of [^{3}H]thymidine into DNA by cell monolayers was determined as in [3]. EGF-stimulation of amino acid transport was determined using α-[^{3}H]aminoisobutyrate [10]. The conditions for receptor radiolabeling and polyacrylamide gel electrophoresis were those in [2,3].

2.2. *Assay of phosphofructokinase in cell extracts*

Cells were suspended (7×10^6 cells/ml) in a hypotonic buffer (10 mM Tris–HCl, pH 7.4), allowed to stand at 0°C for 10 min, homogenized, then centrifuged at 24 000 × *g* for 30 min. The supernate was adjusted to 0.1 mM EDTA/0.1 mM $MgSO_4$/0.1 mM dithiothreitol and used for phosphofructokinase assay. The enzyme was assayed by measuring the disappearance of NADH spectrophotometrically at 340 nm at 25°C [11] by using the reaction conditions in [12]. The reaction mixtures (minus fructose-6-phosphate) were preincubated at 25°C for 10 min. The fall in absorbance was measured 1 min after the addition of fructose-6-phosphate. The reaction rates were generally linear up to ~7 min after substrate addition.

3. Results

3.1. *Effect of MA on the binding of ^{125}I-EGF to cellular receptors at 20°C*

To determine the effect of MA on the average num-

ber of EGF receptor sites per cell and the apparent dissociation constant of the binding reaction, a Scatchard plot of the binding data was prepared (not shown). The number of EGF binding sites per cell was ~130 000 either in the presence or absence of 30 mM MA. The value for dissociation constant (4.9 nM) was also not changed in the presence of MA, suggesting that MA does not appreciably alter the binding characteristics of EGF.

3.2. *Effect of MA on the fate of cell-bound EGF at 37°C*

When cells were first allowed to bind to ^{125}I-EGF at 20°C, and then shifted to 37°C, there was a time-dependent loss of radioactivity from the cells (table 1). In the presence of MA, this loss was considerably reduced (table 1). This loss of cell-bound radioactivity at 37°C is probably due to a combination of the following events: (a) simple dissociation of intact ^{125}I-EGF from the cell surface; and (b) a temperature-dependent internalization of ^{125}I-EGF leading to intracellular degradation, and elimination of the degradation products from the cell [3,5]. In order to differentiate between these two alternatives, we conducted molecular weight analysis of both cell bound and medium radioactivity.

Gel filtration analysis of the radioactivity released into the medium in the absence of MA showed that only a small fraction of the released radioactivity eluted in the excluded volume where intact ^{125}I-EGF eluted, whereas, the labeled material released in the presence of MA was eluted nearly exclusively in the excluded volume (table 1). These results suggest that degradation of EGF (leading to release of degradation products from the cell) is inhibited in the presence of MA. However, the fact that the absolute amount of radioactivity present in the medium in a high molecular weight form is about the same either in the presence or absence of MA (table 1) indicates that the same amount of intact ^{125}I-EGF is dissociated from the cell surface in the presence of MA as in its absence, and this suggests a lack of effect of MA on the internalization process.

To investigate further the effect of MA, we examined the cell-bound radioactivity by gel filtration (fig.1). In the absence of MA at 37°C, there was a time-dependent decrease in the amount of cell-bound high molecular weight form of radioactivity. However, in the presence of MA, a large fraction of the initially bound ^{125}I-EGF radioactivity remained unchanged in the high molecular weight form (i.e., had the same elution volume as intact ^{125}I-EGF) over

Table 1
Effect of MA on the fate of cell-bound ^{125}I-EGF at 37°C

Condition	Incubation at 37°C (h)	Cell-bound radioact. (cpm)	Radioactivity released into medium (cpm)	Released radioact. in high mol. wt. form (%)	Absolute released radioact. in high mol. wt. form (cpm)
Without MA	0	130 000	–	–	–
	0.5	62 000	61 500	47	28 900
	1	51 000	71 000	37	26 300
	2	37 000	95 400	25	23 850
30 mM MA	0	127 000	–	–	–
	0.5	88 000	30 400	78	23 700
	1	80 000	32 100	79	25 400
	2	76 000	37 700	82	30 910

Monolayers of 3T3 cells in 35 mm culture dishes were incubated with ^{125}I-EGF (4.9 nM, 307 500 cpm/ng) with/without 30 mM MA in 1 ml Earles balanced salt solution containing 10 mM Hepes (pH 7.4) and 0.1% bovine serum albumin (EBSS–BSA) at 20°C for 40 min. After washing off unbound radioactivity, 1 ml EBSS–BSA containing either 30 mM MA or no MA was added to each dish and the cells were incubated at 37°C for the indicated time periods. After the supernatant fraction was removed, the cells were solubilized with 0.5 ml NaOH and counted for radioactivity. The supernatant fractions were also counted for radioactivity and then analyzed for intact ^{125}I-EGF content by gel-filtration on a Sephadex G-10 column (0.8 × 20 cm), using portions containing 10 000–40 000 cpm of ^{125}I. The column was equilibrated with 0.15 M NaCl containing 10 mM Tris–HCl (pH 7.4) and 0.1% bovine serum albumin. Fractions of ~0.5 ml were collected. Two peaks of radioactivity were observed, a sharp peak in the excluded volume (fractions 6–9) and a slower eluting diffuse peak. The amount of radioactivity present in fractions 6–9 (corresponding to the elution volume for intact ^{125}I-EGF) was measured and used for calculating the % of released radioactivity in the high molecular weight form

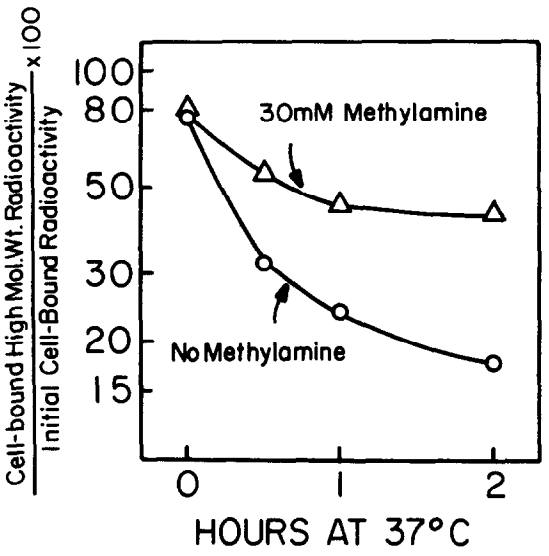

Fig.1. Gel-filtration analysis of cell-bound ^{125}I-EGF radioactivity after incubation at 37°C in the presence and absence of MA. Confluent cultures of 3T3 cells in 35 mm culture dishes were incubated with ^{125}I-EGF (4 nM, 387 900 cpm/ng) in 1 ml EBSS–BSA (containing either 30 mM MA or no MA) at 20°C for 40 min. After washing off the unbound radioactivity the cells were incubated at 37°C with 1 ml EBSS–BSA (containing either 30 mM MA or no MA) for the indicated time periods. After the supernatant fraction was removed, the cell-bound radioactivity was extracted with 1 ml 0.1 M HCl. The acid extracts were neutralized with NaOH, and analyzed by gel filtration as described in the legend for table 1. Total radioactivities (high plus low molecular weight) present in the acid extracts for 0, 0.5, 1 and 2 h incubation in the presence of MA were 132 000, 102 600, 98 700 and 79 700 cpm, respectively. The corresponding radioactivities for 0, 0.5, 1 and 2 h incubation in the absence of MA were 146 000, 88 100, 63 000 and 48 100 cpm, respectively.

0.5–2 h at 37°C (fig.1), suggesting an inhibition of intracellular degradation. The loss observed during 0–0.5 h at 37°C in the presence of MA probably represents simple dissociation of EGF from the cell-surface rather than internal degradation and elimination (see table 1). These results suggest that it is not the internalization of bound EGF, but the degradation of internalized EGF that is blocked by MA.

3.3. *Effect of MA on the fate of receptor after binding to EGF*

We were interested in examining the effect of MA on receptor down regulation [3,5], because an endocytic mechanism may be responsible for this receptor loss. When 3T3 cells were incubated with 1 nM EGF (unlabeled) at 37°C in the absence of MA, and then tested for surface receptor activity by incubating with ^{125}I-EGF at 20°C for 60 min, a 63% receptor loss was observed, with no appreciable change in binding affinity suggesting that this reduction in binding activity is due to an actual loss in the total number of receptor sites, rather than to persistent binding of EGF (fig.2). When an analogous experiment was performed in the presence of MA, we observed that this receptor loss was not diminished but only slightly enhanced (75% loss), with no alteration in binding affinity (fig.2). This ineffectiveness of MA in blocking receptor down regulation was observed not only at the single EGF concentration shown in fig.2, but over a wide range of EGF concentrations (not shown), suggesting the possibility that MA does not block hormone-induced receptor endocytosis.

The effect of MA on intracellular receptor degrada-

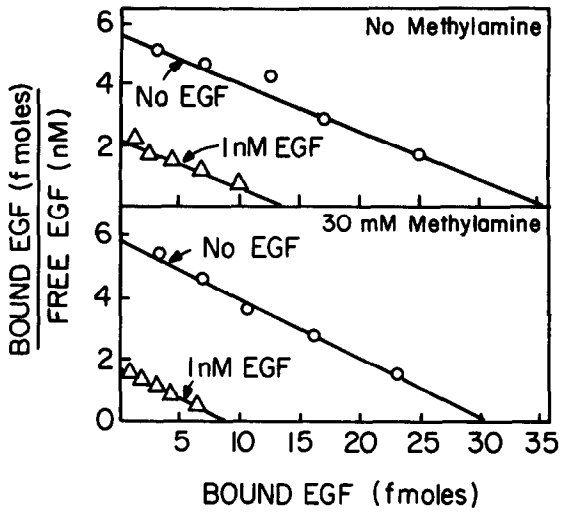

Fig.2. Down regulation of EGF receptor in the absence and presence of MA. Monolayers of 3T3 cells in 16 mm culture dishes were incubated at 37°C for 3 h in Dulbecco's modified Eagles (DME) medium containing 0.1% bovine serum albumin, under the indicated conditions (with/without 1 nM EGF, with/without 30 mM MA). After incubation, each monolayer was washed 5 times with EBSS–BSA using 1 ml for each wash. The washed cells were then incubated at 20°C for 1 h with 0.5 ml EBSS–BSA containing ^{125}I-EGF (210 200 cpm/ng) at varying concentrations (0.59 nM, 1.47 nM, 2.93 nM, 5.9 nM and 14.7 nM). Specific binding of ^{125}I-EGF to the monolayers was determined by measuring the difference in cell-bound radioactivity in the presence and absence of 1 ng unlabeled EGF/ml, and the binding data were subjected to Scatchard analysis.

tion was studied using a covalent radiolabeling technique [2,3]. When cells containing a covalently radiolabeled receptor were incubated at 37°C for varying time periods in medium containing no MA, and then examined by electrophoresis and autoradiography, there was a time-dependent loss of radioactivity from the receptor band, accompanied by appearance of low molecular weight degradation products (detailed [3]). In the presence of MA (5–30 mM), this loss of radioactivity from the receptor band was almost completely prevented (not shown). This action of MA on receptor degradation is very similar to that of chloroquine on EGF receptor metabolism [3]. These experiments, together with the data shown in fig.2, strongly suggest that while MA has no appreciable effect on the internalization of receptor–hormone complexes, the intracellular degradation of these receptor complexes is prevented in the presence of MA.

3.4. *Effect of MA on EGF-induced stimulatory responses*

To determine whether inhibition of hormone–receptor degradation has any effect upon its normal biological functions, we tested for a variety of effects. The concentrations of MA (5–10 mM) used in these experiments were effective in blocking degradation of both EGF and the receptor.

In responsive cells, EGF is known to activate phosphofructokinase, a regulatory enzyme in the glycolytic pathway [12]. The effect of MA on this enzyme is shown in fig.3. Although MA alone could stimulate this basal level of this enzyme in 3T3 cells ~2-fold, it had neither an enhancing nor a suppressing influence upon EGF-stimulation of the enzyme (fig.3). The slight stimulatory effect of MA alone is perhaps due to an elevating influence upon the intracellular pH [13].

The effect of MA upon EGF-induced stimulation of DNA synthesis is shown in fig.4. Incubation of cells with 5 mM MA alone, 1 nM EGF alone, or with 5 mM MA/1 nM EGF for 4 h, resulted in the same degree of stimulation (~1.3-fold). This shows that when the exposure period is short (4 h), MA is by itself stimulatory, but it is neither an enhancer nor a suppressor of EGF-induced stimulation of DNA synthesis. However, when the exposure period is long (10 h), MA no longer stimulates the basal rate, but rather depresses it, and it also inhibits the EGF-stimulated rate of incorporation (fig.4).

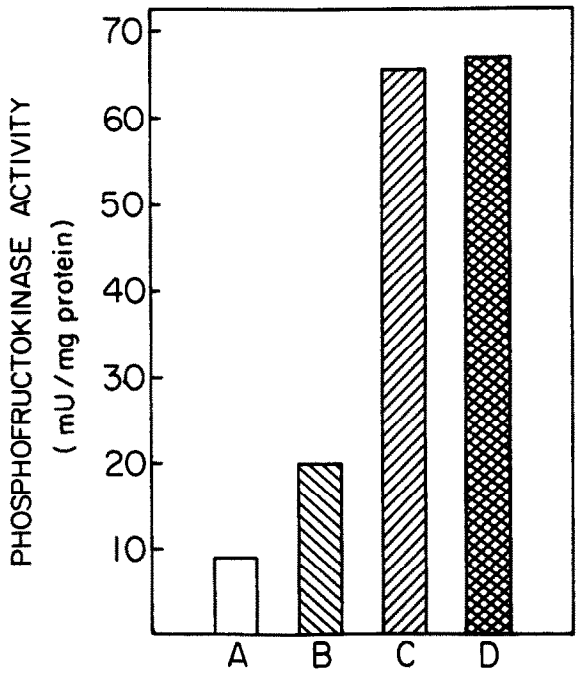

Fig.3. Effect of MA on EGF-induced activation of phosphofructokinase. Monolayers of 3T3 cells in 10 cm dishes were kept for 24 h in a 37°C incubator in 10 ml DME medium containing 1% fetal calf serum. (The medium obtained from these cells at the end of 24 h is referred to as conditioned medium.) At the end of incubation, the cells were washed twice with DME, and then incubated at 37°C for 3 h in conditioned medium containing either no EGF or 2 nM EGF, no MA or 5 mM MA. After this treatment, the monolayers were washed twice with 0.15 M NaCl/10 mM Tris–HCl (pH 7.4) and scraped off the dish using a rubber policeman. Cell extracts were prepared and were analyzed for phosphofructokinase activity with 2.5 mM fructose-6-phosphate (a saturating substrate concentration) as in section 2. A unit of phosphofructokinase is defined as the amount of enzyme required to phosphorylate 1 μmol fructose-6-phosphate/min under the standard conditions in section 2. The following notations have been used for the different treatments undergone by the cells prior to extract preparation: (A) no treatment; (B) 5 mM MA; (C) 2 nM EGF; (D) 5 mM MA and 2 nM EGF.

We also studied the effect of MA upon EGF-induced stimulation of amino acid transport in 3T3 cells (data not shown). A 20% stimulation of α-aminoisobutyrate uptake was observed when cells were incubated with 10 nM EGF for 75 min. This stimulation, although low, is comparable to that obtained (1.2–1.5-fold) with normal EGF responsive cells [10]. Low concentrations of MA (5 mM) had no

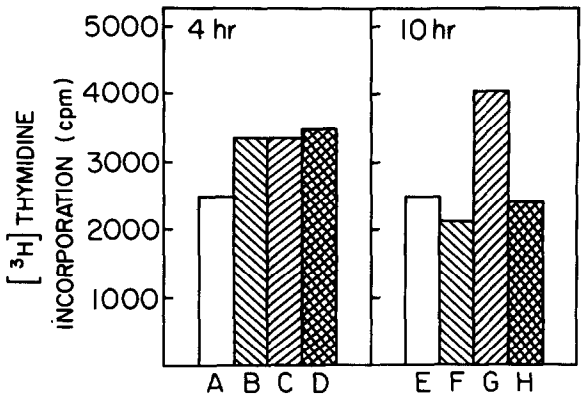

Fig.4. Effect of MA on EGF-induced stimulation of [^{3}H]-thymidine incorporation. Monolayers of 3T3 cells in 16 mm culture dishes were incubated with 1 ml DME medium containing 2% fetal calf serum. (The medium obtained from the cells at the end of 24 h is referred to as conditioned medium.) At the end of incubation 1 nM EGF with or without 5 mM MA in conditioned medium was added to the dishes. After a 4 h or 10 h period of incubation at 37°C, as indicated in the figure, the cells were washed 4 times with conditioned medium, and further incubated at 37°C in conditioned medium containing neither EGF nor MA, for an additional 16 or 10 h, the total incubation time being 20 h. Then [^{3}H]-thymidine (1 μCi/ml, 0.65 μM) incorporation (during a 1 h incubation at 37°C) was measured. The following notations have been used for the different treatments of cells: (A,E) no treatment; (B) 5 mM MA, 4 h; (C) 1 nM EGF, 4 h; (D) 5 mM MA and 1 nM EGF, 4 h; (F) 5 mM MA, 10 h; (G) 1 nM EGF, 10 h; (H) 5 mM MA, 1 nM EGF, 10 h.

effect (stimulatory or inhibitory) on the basal rate of transport, or on the EGF-stimulated transport rate. At higher concentrations of MA (10 mM), there was an inhibition of the basal rate, although the transport system still remained EGF-stimulatable to about the same extent.

4. Discussion

These results strongly suggest that EGF–receptor complexes are internalized and appear to be maintained in an undegraded form in the presence of MA. They agree with the electron microscopic findings in [6] that binding, aggregation and pinocytosis of ferritin-EGF by A-431 human carcinoma cells proceed normally in the presence of MA. The large internal multivesicular structures containing ferritin–EGF–receptor complex did not fuse with lysosomes also, suggesting the occurence of internalization but not degradation [6]. The results in [6] and these biochemical data strongly suggest the occurence of endocytosis in the presence of MA.

There have been conflicting reports on clustering and endocytosis of EGF [7,14]. Using a video intensification microscopic technique, they had observed that clustering and endocytosis of fluorescent analogs of EGF (rhodamine-lactalbumin-EGF conjugate) was inhibited by MA [7]. However these data have been quoted as 'unreliable' in [14]. Using a biochemical method that allows a clear discrimination between cell-surface bound and internalized ^{125}I-EGF, MA had no effect on the internalization of bound ^{125}I-EGF, although it appeared to inhibit the internalization of another ligand, namely α_2-macroglobulin [14]. They concluded that the molecular mechanism of clustering and internalization may differ for these two ligands [14].

Inhibition of degradation of internalized hormone–receptor complexes by MA should prove useful in understanding the role of proteolytic processing in various cellular functions. Studies on the biological properties of leupeptin and colchicine suggest that the inhibitory effects of these compounds on EGF degradation have virtually no effect on the hormone-induced mitogenic response [15,16]. Our results partially accord with their data. We find that the early biological effects of EGF, namely stimulation of amino acid transport, and activation of phosphofructokinase are virtually unaffected by MA, suggesting a lack of involvement of proteolysis in these stimulatory message transmission pathways. On the other hand, stimulation of DNA synthesis which is a late effect of EGF is affected in a time-dependent fashion by MA. There is no potentiating or suppressing influence of MA on EGF-stimulated rate of DNA synthesis when the exposure period is short (4 h). However, there is a stimulatory influence of MA itself on DNA synthesis after this short exposure, agreeing with similar stimulation in [7] 20 h after a 2.5 h exposure to 10 mM MA. With a longer period of exposure (10 h), the situation is reversed. MA alone is no longer stimulatory, and it suppresses completely the EGF-induced stimulation. A possible explanation for this behavior is that prolonged exposures to MA produce certain irreversible damages within the cell (without actually killing the cell), that cause a block in DNA replication and perhaps in other metabolic pathways as well. Another explanation is that perhaps a product of receptor/hormone proteo-

lytic processing inhibited by MA is required for the initiation of the cascade of events that lead to DNA replication, and perhaps the inhibition is reversible when exposure to MA is short, but virtually irreversible after a long period of exposure. Differentiation between these two alternatives is not possible at the present time, as it requires a more rigorous biochemical characterization of the mitogenic message transmission pathway.

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