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EXPOSURE OF CELLS TO AN ACIDIC ENVIRONMENT REVERSES THE INHIBITON BY METHYLAMINE OF THE MITOGENIC RESPONSE TO EPIDERMAL GROWTH FACTOR

A. Christie King and Pedro Cuatrecasas
Department of Molecular Biology
Wellcome Research Laboratories
Research Triangle Park, NC 27709

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SUMMARY. Lysosomotropic alkylamines inhibit the mitogenic response of cells to epidermal growth factor. This inhibition can be reversed if the extracellular medium is acidified for a brief period of time. More importantly, the period of time that EGF is required in the medium to elicit responses is reduced from 8 hours to only 4 hours. The response is specific since antisera against epidermal growth factor prevent the enhancement in DNA synthesis. The response is dependent on the external pH and the duration of the exposure. Thus, stimulation of cultured fibroblasts by epidermal growth factor may require exposure to an acidic compartment like that found in lysosomes, and supports the notion that inhibition of mitogenesis by alkylamines is caused by their ability to raise the pH in an intracellular acid compartment.

INTRODUCTION. Primary and tertiary alkylamines are potent inhibitors of growth potentiation mediated by epidermal growth factor (EGF), insulin, and serum (1). These amines are known to produce lysosomal dysfunction by raising the pH of intracellular acid compartments (2). Thus, in their presence, EGF degradation is inhibited by about 95% (3,4), while binding and internalization of ^{125}I-EGF proceed unchanged (4,5,6). Interestingly, the concentrations of various alkylamines that induce maximal lysosomal pH changes (2), and thus inhibit degradation of EGF (3,4), correlate well with those that inhibit mitogenesis (1).

In light of the profound inhibitory effects of alkylamines on the induction of mitogenesis, we postulated that processing of the EGF-receptor complex in lysosomes might produce an activated product capable of direct interaction with nuclear components which would subsequently initiate DNA synthesis (1). Since other agents (leupeptin and antipain) can inhibit degradation of EGF

Abbreviations: EGF, epidermal growth factor; Dp, Diphtheria; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum.

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with little or no inhibitory effect on the DNA synthetic response (7,8), it seems likely that the "processing" required for activity may not be simple lysosomal degradation of the complex. It is pertinent that lysosomotropic drugs prevent certain enveloped viruses from infecting mammalian cells (9,10) and can also inhibit the cytotoxic action of diphtheria (Dp) toxin (11-14). In these cases the inhibitory effects of amines are readily reversed by exposing cells to a medium of reduced pH, and there is evidence that under external acidic conditions the normal endocytic pathway may be bypassed (9-14). For these reasons, we have studied whether exposure of the EGF-receptor complex to an acidic environment like that of lysosomes may allow production of a mitogenic response even when alkylamines are present in the incubation medium.

MATERIALS AND METHODS. Human fibroblast explants (A34) were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) as described earlier (1,6). The night before an experiment, the medium was changed to DMEM, 0.5% FBS, 20 mM Hepes, pH 7.2, to deprive the cells of serum and further assure growth arrest. The rate of DNA synthesis in cultures was determined by incubating cells with 1 μCi/ml [^{3}H-methyl thymidine for 4 hours after the treatments described in the figure legends. The amount of DNA synthesized was determined as described previously (1).

RESULTS AND DISCUSSION. Since lysosomotropic drugs raise the pH within intracellular acid compartments (2), their effect on EGF action (1) could possibly be explained by alterations in the intralysosomal pH. To study this, we exposed cells to 10 ng/ml EGF for 4 hours or 20 hours in the presence or absence of 15 mM $MeNH_2$ (Figure 1). The characteristic increase in DNA synthesis observed after incubation with EGF for 20 hours (a and b) was not observed when 15 mM $MeNH_2$ was present (c). In parallel incubations, cells were exposed to 10 ng/ml EGF with 15 mM $MeNH_2$ for 4 hours, after which the medium was changed and the cells were incubated in medium at pH 5.8 for 5 minutes at 4°. The medium was then changed back to pH 7.2 without EGF and the incubation was continued for another 16 hours. All washing and incubation buffers contained 15 mM $MeNH_2$ to ensure that degradation of EGF was blocked throughout the experiment (3,4,6). When cells were subjected to this brief incubation at reduced pH, two important observations were made. Firstly, and most significantly, a mitogenic response was elicited even after exposure of cells to EGF

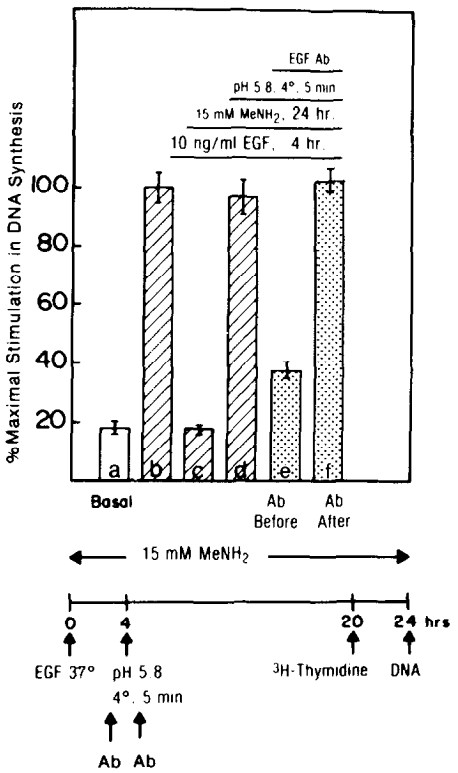


Figure 1. Effect of $MeNH_2$ and Low pH on Mitogenic Activity of EGF. Confluent and serum-starved cells were untreated (a) or were exposed to 10 ng/ml EGF for 24 hours (b,c) or for 4 hours (d-f). Some cells were incubated with 15 mM $MeNH_2$ throughout the experiment (c-f), and some were exposed to pH 5.8 medium for 5 minutes at 4° (d-f). The experimental protocol for columns d-f is shown at the bottom of the figure. When the EGF antisera was added before the pH change (e), it was only present during the 5 minute, pH 5.8 incubation. When it was added after the pH change (f), it remained in the incubation medium throughout the duration of the experiment. Briefly, cells were exposed to 10 ng/ml EGF for 4 hours, 37° with 15 mM $MeNH_2$. Afterwards, the cells were washed twice with ice-cold DMEM, 0.5% FBS, 15 mM $MeNH_2$, 20 mM Hepes, pH 7.2, and then incubated for 5 minutes in DMEM, 0.1% BSA, 15 mM $MeNH_2$, 20 mM MES, pH 5.8. The cells were washed twice again in pH 7.2 medium and then incubated for a further 12 hours in the same medium prior to the addition of ^{3}[H]-thymidine.

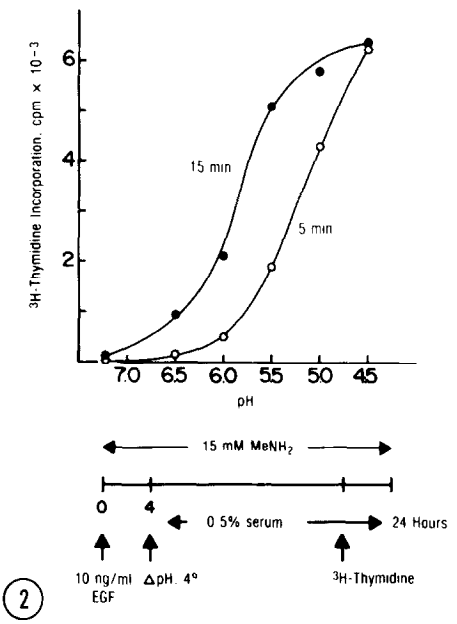
for a period of time (4 hr) which is usually insufficient to evoke increased DNA synthesis (1,14-16). Secondly, the inhibitory effect of $MeNH_2$ on the mitogenic response of A34 cells to EGF was completely reversed (d).

To ensure that the response measured was specific to EGF, we determined whether the increased synthesis of DNA could be prevented by antisera to EGF (14,15). Cells were treated under conditions identical to those described above, except that 10 μg/ml of EGF antibody was added either before or after the incubation at pH 5.8. When the anti-EGF was added before the pH change, the DNA synthetic response was inhibited by about 80% even though the antibody

was present only during the pH 5.8 incubation (e). However, when the antibody was added after the pH shift, no inhibition was observed (f). These results suggest that EGF was accessible to inactivation by the antibody only when it was added before the pH change. Thus, the DNA synthetic response was specific to EGF, but after the cells were exposed to the acidic environment, the mitogen was masked from the anti-EGF and the cells were committed to respond.

To further characterize the effect of an acidic environment on EGF-induced stimulation of DNA synthesis, we repeated the incubation with EGF and $MeNH_2$ as described in Figure 1, but exposed cells to medium at different pH values (pH 4.5 to 7.2) for either 5 or 15 minutes at 4° (Figure 2). The response to EGF increased as the pH of the medium became more acidic, with a maximal response at pH 4.5 for both incubation times. The half-maximal response is shifted to more basic pH values when cells are exposed to reduced pH for 15 minutes (pH 5.9) as compared to a 5 minute incubation (pH 5.2). Exposure of A34 cells to reduced pH had no effect on the basal rate of DNA synthesis until the pH values were reduced below 5.0. At these levels, some cells were lost from the plates (not shown), and the basal rate of synthesis was reduced. Nonetheless, a significant increase in ^{3}H-thymidine incorporation over basal was observed when EGF was present.

Since only the pH of the extracellular medium was changed and $MeNH_2$ was present throughout the duration of the experiments, we assume that no cell-associated EGF was degraded. In support of this, we find that under the conditions of the assay, no ^{125}I-EGF is released from cells (Figure 3). This is consistent with a lack of EGF degradation (3,4) and with trapping of ^{125}I-EGF-receptor complexes into cytoplasmic vesicles (4,5) when MeNH is present. These results may indicate that residual EGF-receptor complexes at the cell surface can be "activated" even when $MeNH_2$ is present by briefly exposing cells to an acidic environment. Thus, the possibility arises that the normal activation pathway may include a critical exposure of the EGF-receptor complex to an intracellular acid compartment. The most likely site would be lysosomes, where 50 to 90% of the EGF is degraded (3,4,6,16), although it is not possible to exclude the



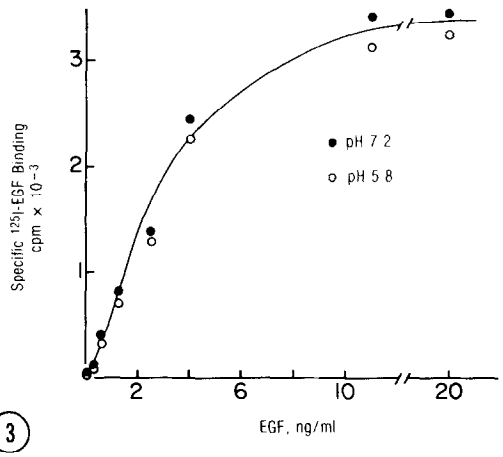


Figure 2. Effect of pH and Duration of Low pH Exposure on Mitogenic Response. Cells were incubated as described in figure 1, column d except that the pH was varied during the 4° incubation and the cells were incubated for 15 minutes at the reduced pH values as well as for 5 minutes.

Figure 3. Exposure of Cells to Low pH Does Not Cause Dissociation of Cell-Associated, ^{125}I-Labeled EGF. Cells were incubated with $MeNH_2$ and ^{125}I-labeled EGF for 4 hours before exposure to pH 5.8 (o) or pH 7.2 (•) for 5 min at 4° (as in Figures 1 and 2). The cells were then washed and counted for radioactivity.

participation of a non-lysosomal, endocytic compartment having a low intravesicular pH. The present experiments suggest that simulation of an acid environment normally encountered by the mitogen as it enters an endosomal compartment may be sufficient to overcome the inhibitory effects of alkylamines. It is thus possible that the "activation" pathway may compete with the degradative (lysosomal) process, and that the pathway for cellular activation by EGF is normally extremely inefficient. This could possibly account for the prolonged periods of EGF exposure required to affect mitogenic responses (14-16).

Lysosomotropic alkylamines also inhibit the ability of Dp toxin to prevent protein synthesis in cultured cells (11-13) and the infectious processes of a variety of eukaryotic, enveloped viruses in vitro (9,10). Both toxins and enveloped viruses require endocytic entry into the cytoplasm of mammalian cells in order to exert their biological effects (9-13). These agents are now

thought to enter cells via receptor-mediated processes, and they normally do not gain access directly through the plasma membrane (9). The pathway for internalization of Dp toxin and viruses is similar to that of EGF, in that all accumulate in coated pits which invaginate and form cytoplasmic vesicles which fuse with lysosomes and, like EGF, are degraded (9,12,13). The inhibitory effects of alkylamines on virus infectivity and toxin action can also be overcome by briefly exposing cells to a medium of reduced pH (9,12,13). In these studies, it was suggested that the entry of both eukaryotic viruses and Dp toxin into the cellular cytoplasm is through the lysosomal compartment and that the pH gradient between the lysosome and cytoplasm is necessary for ejection of the biologically active component out of the lysosome and into the cytoplasm (9-13). Our studies suggest that the activation pathway for EGF may share certain features in common. Under conditions where the pH of the medium is lowered, the endocytic pathway is presumably bypassed and a toxin fragment, viral contents or component of the EGF-receptor complex can penetrate the plasma membrane and enter directly into the cytoplasm.

To our knowledge, this is the only report that demonstrates a significant reduction in the usual EGF exposure period required to produce a mitogenic response. It is therefore provocative to speculate that lysosomal or other endocytic compartmentalization may be a critical step in the pathway to mitogenesis, and that in a fashion similar to that which occurs with D_p toxin, a mitogenic fragment of the EGF-receptor complex may be generated which is somehow extruded from the vesicles into the cytoplasm by a pH-sensitive mechanism

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