Activity of the epidermal-growth-factor receptor and phospholipase C- γ 1 in heat-stressed fibroblasts and A-431 cells

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A variety of changes in the functions of specific plasma-membrane components have been reported in cells exposed to a heat shock. In this study, we examined the consequences of heat stress on epidermal-growth-factor (EGF)-induced receptor autophosphorylation and receptor-mediated tyrosine phosphorylation of phospholipase C- γ 1 (PLC- γ 1), a cellular substrate. Although the tyrosine kinase activity of the EGF receptor is rapidly inactivated at 45 °C *in vitro* [Carpenter, King & Cohen (1979) J. Biol. Chem. **254**, 4884–4891], EGF stimulates autophosphorylation of its receptor in both A-431 cells and human fibroblasts after a prolonged heat shock. Phosphoamino acid analysis of the receptor reveals an EGF-induced increase in phosphotyrosine and phosphoserine at 46 °C. EGF also stimulates the phosphorylation of phospholipase C- γ 1 and induces the formation of inositol phosphates under heat-shock conditions. 125 I-EGF binding and internalization in A-431 cells is not decreased during incubations at 46 °C for up to 90 min. EGF-induced dimerization of EGF receptors on the cell surface is preserved during heat shock. Though EGF-receptor-mediated endocytosis is not inhibited by elevated temperature, the degradation of internalized 125 I-EGF is dramatically decreased. These results indicate that, aside from ligand degradation, the EGF-mediated pathway of signal transduction through phospholipase C- γ 1 remains remarkably intact during conditions of extreme cellular stress.

INTRODUCTION

The responses of individual cells to stress in their micro-environment are the basis of a complex organism's ability to withstand systemic offences. Exposure of cells to a rapid increase in environmental temperature, termed 'heat-shock', has been used to identify numerous cellular changes that occur in response to stress in general [1]. The heat-shock response, now generally termed the stress response, occurs in all organisms thus far examined. Many of the changes associated with the cellular stress response have been extensively studied, including the marked decrease in general protein synthesis, the induction of heat-shock-protein synthesis, and morphological changes [2,3]. Less extensively studied have been the effects of heat stress on intrinsic cellular regulatory pathways. Also, little is known about the intracellular systems that may signal induction of components of the stress response.

Although most investigations of the stress response have focused on the expression of stress-response genes, there are several reports describing early biochemical changes associated with the plasma membrane. Insulin binding is decreased when HA-1 cells are incubated at 43 °C, owing to a decrease in the number of cell-surface insulin receptors [4]. Hyperthermia induces a decrease in the expression of specific mouse major histocompatibility antigens at the cell surface [5], and also affects Na⁺/K⁺-ATPase activity such that intracellular K⁺ levels are increased [6,7]. However, few studies of the influence of heat stress on growth-factor-mediated signal-transduction systems have been reported.

The epidermal-growth-factor (EGF) receptor is a 170 kDa integral plasma-membrane protein that specifically binds its cognate ligand, EGF, and related molecules [8]. After formation of EGF-receptor complexes, both ligand and receptor are internalized and ultimately degraded in lysosomes [9–12]. The receptor has a cytoplasmic tyrosine kinase domain, which is

activated upon EGF binding to the extracellular domain [13]. The activated EGF receptor phosphorylates four tyrosine residues near its C-terminus [14–16], and several intracellular proteins as well.

Perhaps the most characterized receptor-mediated cellular substrate is phospholipase C- γ 1 (PLC- γ 1). PLC- γ 1 mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP₂), producing 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (InsP₃). Diacylglycerol may activate protein kinase C [17], whereas InsP₃ regulates intracellular Ca²⁺ levels by inducing the release of stored Ca²⁺ from endoplasmic reticulum [18]. EGF, as well as several other growth factors, is able to bring about rapid increases in phosphotyrosine and phosphoserine in PLC- γ 1 [19–22]. Direct evidence of an increase in the catalytic activity of PLC- γ 1 owing to growth-factor-stimulated tyrosine phosphorylation has been demonstrated *in vitro* [23,24] and *in vivo* [25]. In the present paper, we examine the effects of heat shock on the EGF-induced phosphorylation and activation of phospholipase C- γ 1. Since the EGF-receptor tyrosine kinase is rapidly inactivated at 45 °C *in vitro* [26], we have also sought to determine whether this tyrosine kinase is attenuated by heat stress in intact cells. Two cell lines were used for these studies: A-431 epidermoid carcinoma cells, which contain an exaggerated number of EGF receptors (approx. 2×10^6 receptors per cell [27]) and non-transformed human fibroblasts, which have a moderate complement of EGF receptors (approx. 5×10^4 per cell).

MATERIALS AND METHODS

Materials

EGF was isolated from mouse submaxillary glands [28], and iodinated [9] as previously described. [32 P]P_i, 125 I-labelled goat anti-mouse IgG, 125 I-Protein A and monoclonal antibodies to phosphotyrosine (PY20, PY69) were obtained from ICN

Abbreviations used: EGF, epidermal growth factor; HF, human fibroblast; PLC- γ 1, phospholipase C- γ 1; DMEM, Dulbecco's Modified Eagle's Medium; BS³, bis(sulphosuccinimidyl) suberate; TCA, trichloroacetic acid.

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Biomedical (Irvine, CA, U.S.A.). Polyclonal antibody to phosphotyrosine was obtained from Zymed (San Francisco, CA, U.S.A.). *myo*-[2-³H]inositol was obtained from New England Nuclear. Cross-linking reagents were purchased from Pierce.

Cell culture

Cultures of human fibroblasts [29] and A-431 cells [30] were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO) containing 20 mM-Hepes, pH 7.4 (Calbiochem), and 50 μ g of gentamycin (Sigma)/ml. Media for A-431 cells was supplemented with 10% (v/v) calf serum, whereas 10% fetal-bovine serum was added to human fibroblast (HF) cultures. For most experiments, A-431 cells were grown in 60 mm dishes (Costar) until about 75% confluent (approx. 8×10^4 cells/cm²). Human fibroblasts were grown in 100 mm dishes (Costar) until approx. 75% confluent.

Metabolic labelling

A-431 or HF cells were washed twice with phosphate-free modified Eagle's medium (MEM-PF), 3 ml of MEM-PF was added to each dish, and 500 μ Ci of [³²P]P_i/ml was then added. Cells were incubated for 3–4 h at 37 °C with 5% CO₂ before heat-stress induction. For immunological detection of phosphotyrosine, cells were pre-washed twice with DMEM containing 10% dialysed calf serum. Dishes containing DMEM + 10% dialysed calf serum were then incubated in a 37 °C incubator with 5% CO₂ for at least 1 h before heat-shock treatment.

Induction of heat stress and EGF stimulation

For most experiments, cells preincubated at 37 °C were rapidly transferred to an adjacent humidified incubator containing 5% CO₂ and a water-bath pre-equilibrated at 46 °C. Culture dishes were placed directly on to the water-bath to induce heat-shock conditions. Cells were thus subjected to a sudden and rapid increase in temperature, while a 5%-CO₂ environment was maintained. The CO₂ environment was important for cell survival. To initiate growth-factor responses, 20 (HF cells) or 100 (A-431 cells) ng of EGF/ml was added to the culture medium during the final 5–10 min of incubation at 46 °C. The culture dishes were then placed on ice, carefully and rapidly washed twice with ice-cold Ca²⁺/Mg²⁺-free phosphate-buffered saline (140 mM-NaCl/3 mM-KCl/9 mM-Na₂HPO₄/1.5 mM-KH₂PO₄), and solubilized for 15 min at room temperature with 750 μ l of TGP buffer (1% Triton X-100, 10% glycerol, 20 mM-Hepes, pH 7.4, 1 mM-sodium orthovanadate and 1 mM phenylmethanesulphonyl fluoride).

Immunoprecipitation and immunoblotting

Radiolabelled extracts were pre-cleared with *Staphylococcus aureus* cells (Behring Diagnostics) before immunoprecipitations. Equal amounts of cell extract were then incubated with anti-EGF-receptor antibody 986 [31] or anti-PLC- γ 1 antibody [32]. Immunoprecipitates were washed four times with RIPA buffer (50 mM-Tris, pH 8.5, 150 mM-NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 0.02% Na₂S₂O₃) and subjected to SDS/PAGE on 7.5%-polyacrylamide gels [33].

For Western-blot experiments, non-metabolically labelled cells were washed and solubilized in the same manner and the extracts subjected to SDS/PAGE. Proteins were then transferred to nitrocellulose, which was subsequently washed in a blocking buffer (50 mM-Tris, pH 8.0, 2 mM-CaCl₂, 80 mM-NaCl, 0.2% Nonidet P-40 and 5% non-fat dry milk). Blots were probed with either a 1:1 mixture of mouse anti-phosphotyrosine antibodies

PY69 and PY20 (final dilution of 1:3300) or polyclonal anti-phosphotyrosine antibody (final dilution 1:2000). Bound antibodies were detected with ¹²⁵I-labelled goat anti-mouse IgG or ¹²⁵I-Protein A.

Phosphoamino acid analysis

Cells were labelled with [³²P]P_i, and subjected to heat treatment and growth-factor stimulation. The cells were then solubilized, immunoprecipitated, and subjected to SDS/PAGE as described above. Radiolabelled proteins were then transferred to Immobilon paper (Millipore) and autoradiography was performed. After the ³²P-labelled EGF receptor and PLC- γ bands were excised from the Immobilon and the protein was digested by acid hydrolysis [34], the ³²P-labelled phosphoamino acids were separated by thin-layer electrophoresis (1 kV, 60 min) in a pyridine/acetic acid/water (1:10:189, by vol.) buffer, pH 3.5 [34]. The positions of the radiolabelled phosphoamino acids were compared with those of unlabelled phosphotyrosine, phosphoserine and phosphothreonine (Sigma).

Analysis of inositol phosphates

A-431 cells in six-well dishes (Costar) were labelled with 2 μ Ci of *myo*-[2-³H]inositol/ml for 48–72 h. Cells were washed twice with warm DMEM containing 0.1% BSA and 20 mM-LiCl. Culture dishes were then heat-treated for 30 min in the same medium containing 2 μ Ci of [³H]inositol/ml. EGF was added to the appropriate wells for the last 5 min of the heat shock. The medium was removed at the indicated times, and soluble inositol phosphates were extracted with 1 ml of ice-cold 10% (v/v) trichloroacetic acid (TCA). The TCA-precipitated extracts were centrifuged (12 000 *g* for 2 min), and the supernatants collected. After ether extractions, the inositol phosphates were separated by elution from a Dowex formate resin column [35]. The columns were washed as previously described [36].

¹²⁵I-EGF binding, internalization and degradation

A-431 cells were washed twice with DMEM plus 0.1% BSA and preincubated at 37 °C or 46 °C for 10 min. ¹²⁵I-labelled EGF (sp. radioactivity approx. 93 000 c.p.m./ng) at a final concentration of 10 ng/ml was added to the cells, which were then incubated for the indicated periods of time. Non-specific binding was determined by addition of a 100-fold excess of unlabelled EGF to replicate wells. Internalization of ¹²⁵I-EGF was determined by the acid-wash procedure [37].

To assay ¹²⁵I-EGF degradation, cells were washed twice with Hanks' balanced salt solution at room temperature before incubation in DMEM containing 0.1% BSA. A solution containing ¹²⁵I-EGF (final concn. 10 ng/ml) was added immediately after transfer to a water-bath pre-warmed to 46 °C. After a 30 min incubation, the cell monolayers were gently but rapidly washed four times with pre-warmed (46 °C) 50 mM-glycine/100 mM-NaCl buffer, pH 4.0, to remove surface-bound and unbound radioactivity [37]. DMEM containing 0.1% BSA was then re-added to the dishes, and the cells were incubated at 46 °C for an additional 90 min. To assay for released intact and degraded ¹²⁵I-EGF, the conditioned medium was removed and subjected to acid precipitation with 10% trichloroacetic acid/1% phosphotungstic acid solution (1:2, v/v) for 18 h at 4 °C [38]. The precipitated proteins, including intact ¹²⁵I-EGF, were pelleted by centrifugation at 2000 *g* for 10 min. Radioactivity in the non-TCA-precipitable supernatants, containing ¹²⁵I-labelled products of EGF degradation, was determined with a γ -radiation spectrometer. The remaining cells were solubilized with TGP buffer for 10 min at room temperature, and subjected to acid precipitation, as des-

cribed above, to determine the presence of intracellular intact or degraded ^{125}I -EGF.

EGF receptor dimerization

A-431 cells were incubated in Hanks' balanced salt solution at 4, 37 or 46 °C for 10 min before addition of EGF (100 ng/ml). The membrane-impermeant bifunctional cross-linking reagent bis(sulphosuccinimidyl) suberate (BS^3) [39] was then added for 20 min to a final concentration of 2 mg/ml. The cross-linking reaction was then stopped with 100 mM-glycine. The cells were solubilized with TGP buffer, and the extracts prepared for SDS/PAGE on a 3–10 % gradient gel. After transfer of proteins to nitrocellulose, the blots were incubated with anti-EGF-receptor antiserum, washed, and bound antibody was detected with ^{125}I -Protein A.

RESULTS

EGF-stimulated receptor phosphorylation during heat shock

To determine whether heat stress alters EGF receptor functions, A-431 cells were exposed to a rapid temperature increase from 37 °C to 46 °C. As determined by Trypan Blue exclusion, more than 90 % of cells survived the heat treatment for 2 h (S. M. Liu, unpublished work). This stress induced elements of a classic heat-shock response in these cells, including a marked decrease in general protein synthesis as determined by [^{35}S]methionine labelling (results not shown).

When A-431 or HF cells metabolically labelled with [^{32}P]P_i are incubated at 46 °C for 30 min and EGF is added for the final 5 min, the growth factor strongly stimulates phosphorylation of the EGF receptor (Fig. 1a). The increase in the extent of EGF-induced phosphorylation of the receptor in A-431 cells at 46 °C is comparable with that at 37 °C. In HF cells, the increase in EGF-stimulated receptor phosphorylation was also comparable at 37 °C and 46 °C. In these cells, however, there is also an increase in the basal receptor phosphorylation during the heat shock (Fig. 1a, lane 7). The level of phosphotyrosine associated with the EGF receptor increases upon EGF addition [40]. Immunoblot analysis with phosphotyrosine antibodies (Fig. 1b) shows that EGF stimulates tyrosine phosphorylation of the receptor in A-431 and HF cells that have undergone a 45 min heat shock at 46 °C before addition of EGF. After EGF stimulation of A-431 cells at 37 °C or 46 °C, phosphoamino acid analysis of ^{32}P -labelled EGF-receptor protein shows that EGF significantly increases phosphotyrosine and phosphoserine and produces smaller increases in phosphothreonine (Fig. 2).

The increased basal phosphorylation of the receptor during heat stress (Fig. 1a, lane 7) is not due to increases in receptor biosynthesis and/or cell-surface expression. EGF-receptor protein synthesis, reflecting general protein synthesis, is drastically decreased during heat shock. Immunoblot analysis with anti-EGF-receptor antibody reveals that the amount of receptor remains unchanged during 2 h of heat stress (results not shown). Also, the EGF-induced phosphorylation of the receptor at 46 °C is not dependent on heat-shock-protein synthesis during the heat stress. When A-431 or HF cells, pre-treated with 10 μg of cycloheximide/ml for 30 min, are heat-shocked and then treated with EGF, the ligand-dependent increase in phosphotyrosine content of the EGF receptor is maintained (Fig. 1c).

EGF-stimulated PLC- γ 1 phosphorylation during heat shock

Since the γ -1 isoform of phospholipase C has been shown to be a substrate for the activated EGF receptor kinase [19–22], we determined whether this signal-transduction event occurs in cells subjected to a heat stress. Cells were labelled with [^{32}P]P_i, placed at 37 °C or 46 °C for 30 min, and EGF was added for the last

5 min of the incubation. Immunoprecipitation of PLC- γ 1 from heat-shocked A-431 or HF cells demonstrates that EGF stimulates PLC- γ 1 phosphorylation at 46 °C (Fig. 3). The ligand-induced increase in phosphorylation of PLC- γ 1 during heat shock in A-431 cells is slightly less (30 %) than at 37 °C. This difference is attributable to smaller increases in phosphoserine and phosphotyrosine on PLC- γ 1 with EGF stimulation at 46 °C compared with 37 °C (results not shown).

Fig. 1. EGF-stimulated receptor phosphorylation during heat shock

(a) Total ^{32}P incorporation into the EGF receptor. A-431 cells or HF cells were pre-labelled with [^{32}P]P_i and incubated at 37 °C, or heat-shocked at 46 °C, for 30 min. EGF (100 or 20 ng/ml) was added for the last 5 min of incubation. Cell lysis and immunoprecipitation of EGF receptor were performed as described in the Materials and methods section. (b) EGF receptor tyrosine phosphorylation at 46 °C. A-431 and HF cells were subjected to 45 min of heat stress at 46 °C. EGF was added to the appropriate dishes (100 ng/ml, lanes 2 and 4; 20 ng/ml, lanes 6 and 8) for the last 10 min of incubation before SDS/PAGE and Western blotting. The blot was probed with a 1:1 mixture of anti-phosphotyrosine antibodies PY69 and PY20, and made visible by using ^{125}I -labelled goat anti-mouse IgG. (c) Influence of cycloheximide on EGF receptor phosphorylation at 46 °C. A-431 cells were treated with 10 μg of cycloheximide/ml for 30 min at 37 °C before transfer to 46 °C for a 30 min heat-stress treatment in the presence or absence of EGF during the last 5 min of incubation. Western-blot analysis was performed with phosphotyrosine antibodies as described above.

Fig. 2. Phosphoamino acid composition of EGF receptor from heat-stressed A-431 cells

A-431 cells were metabolically labelled with [^{32}P]P_i and heat-shocked at 46 °C for 30 min in the absence (lane 3) or presence (lane 4) of 100 ng of EGF/ml added for the last 5 min of the incubation. Control cells were incubated at 37 °C, in the absence (lane 1) or presence (lane 2) of 100 ng of EGF/ml. After cell solubilization, SDS/PAGE and transfer to Immobilon paper, ^{32}P -labelled EGF receptor protein bands were excised, and phosphoamino acid analysis was performed as described in the Materials and methods section. Radioactive spots corresponded to locations of the unlabelled phosphoamino acid standards: phosphoserine (PS), phosphothreonine (PT) and phosphotyrosine (PY).

Fig. 3. PLC- γ 1 phosphorylation at 46 °C

A-431 or HF cells were labelled with [^{32}P]P_i for 3 h and then incubated at 37 °C or 46 °C; 100 ng of EGF/ml was added to A-431 cells, and 20 ng of EGF/ml was added to HF cells, for the last 5 min of incubation. PLC- γ 1 was immunoprecipitated and analysed by gel electrophoresis and autoradiography as described in the Materials and methods section.

EGF-stimulated inositol phosphate formation during heat shock

To determine if the phosphorylation of PLC- γ 1 in EGF-treated heat-shocked cells leads to increased hydrolysis of phosphoinositides, we measured the accumulation of inositol phosphates after EGF addition. EGF (200 ng/ml) was added for 5 min to [^3H]inositol-prelabelled A-431 cells that had been heat-shocked at 46 °C for 25 min. Control dishes were subjected to

Fig. 4. Inositol phosphate formation in heat-stressed A-431 cells

A-431 cells were metabolically labelled with [^3H]inositol for 48–72 h. The cells were then incubated at 37 °C or 46 °C for 30 min, in the absence (□) or presence (▨) of 200 ng of EGF/ml added for the last 5 min. Levels of ^3H -labelled inositol phosphates formed after this treatment were determined as described in the Materials and methods section. IP₁ refers to the total amount of ^3H -labelled inositol phosphates formed, and is equivalent to InsP₁ + InsP₂ + InsP₃ in each specific condition.

46 °C heat shock for 30 min without EGF addition or were incubated at 37 °C in the absence or presence (5 min) of EGF. The data in Fig. 4 indicate that there is no significant difference in the basal amounts of inositol phosphates in A-431 cells incubated at 37 °C or 46 °C. When EGF is added, the accumulations of inositol monophosphate (InsP₁) (Fig. 4a) and inositol bisphosphate (InsP₂) (Fig. 4b) are increased 2-fold and 3-fold, respectively, at both 37 °C and 46 °C. The EGF-provoked increases in inositol trisphosphate (InsP₃) are 45 % and 35 % for cells incubated at 37 °C and 46 °C respectively (Fig. 4c). Overall there is a 2-fold stimulation by EGF of total inositol phosphates in heat-stressed cells (Fig. 4d), comparable with the 2-fold increase observed in cells maintained at 37 °C. This suggests that PLC- γ 1 remains functional and able to be activated by EGF treatment of cells exposed to heat-shock conditions.

^{125}I -EGF binding and receptor dimerization in heat-stressed cells

Since substrates of the EGF receptor are phosphorylated in cells subjected to heat shock, we performed binding studies with ^{125}I -EGF to determine whether heat stress influences ligand-receptor interactions. A-431 cells were placed at 46 °C and ^{125}I -EGF (final concn. 10 ng/ml) was added to wells immediately after commencement of the heat shock. Cells were then incubated for up to 90 min to determine the time course of ligand binding. No difference was noted in ^{125}I -EGF binding to control (37 °C) and heat-stressed (46 °C) cells (Fig. 5a).

A rapid response to EGF-receptor binding of ligand is the formation of receptor dimers [41]. We determined whether heat

Fig. 5. Effect of heat shock on ^{125}I -EGF binding and receptor dimerization

(a) Effect of heat shock on ^{125}I -EGF binding to A-431 cells. A-431 cells were incubated at 37°C (○) or 46°C (●) and 10 ng of ^{125}I -EGF/ml was added. At the indicated time points, cells were assayed for the amount of cell-associated radioactivity, as described in the Materials and methods section. (b) EGF receptor dimerization during heat shock. A-431 cells were incubated at 4°C, 37°C or 46°C for 10 min before addition of 100 ng of EGF/ml and 2 mg of BS³/ml. Incubations were continued for an additional 20 min before cell extracts were prepared and electrophoresed on 3–10% SDS/polyacrylamide gradient gels. Western blotting was performed by using anti-EGF-receptor antibody and ^{125}I -Protein A.

Fig. 6. Internalization and degradation of ^{125}I -EGF during heat stress

(a) ^{125}I -EGF internalization in heat-shocked A-431 cells. Cells were incubated at 37°C or 46°C for 10 min, 10 ng of ^{125}I -EGF/ml was added, and then cells were incubated for the indicated times. Internalized ^{125}I -EGF was determined by the acid-wash procedure noted in the Materials and methods section. The amount of ^{125}I -EGF internalized by cells at 37°C (○) or 46°C (●) is plotted. (b) ^{125}I -EGF degradation in heat-shocked A-431 cells. Cells were treated with 10 ng of ^{125}I -EGF/ml for 30 min at 37°C or 46°C. After washes with a pH 4.0 buffer, the cells were allowed to incubate for a further 90 min at 37°C or 46°C. Total radioactivity (■), and non-TCA-precipitable radioactivity (□) containing ^{125}I -EGF degradation products, were determined from conditioned medium and solubilized cells as described in the Materials and methods section.

stress affects this process. A-431 cells were heat-shocked in Hanks' balanced salt solution for 10 min before addition of EGF and the cross-linker BS³. After an additional 20 min at 46°C, the cross-linking reaction was terminated. The cells were then harvested, solubilized, and proteins in the cell extract separated by SDS/PAGE and transferred to nitrocellulose paper. EGF receptors were detected by Western blotting. The data in Fig. 5(b) show that ligand-induced dimerization of EGF receptors occurs under heat-shock conditions. The extent of receptor dimer formation at 46°C is similar to the dimerization that occurs at 37°C. This indicates that the mechanism by which EGF receptors are induced to aggregate in the plasma membrane is not inhibited by heat stress.

^{125}I -EGF internalization and degradation in heat-stressed cells

After EGF binding to surface receptors, the complexes are internalized by receptor-mediated endocytosis [9–12]. A-431 cells were incubated at either 37°C or 46°C, ^{125}I -EGF was immediately added, and the incubation continued for the indicated times. Surface-bound and internalized ^{125}I -EGF were determined by the acid-wash technique [37]. Fig. 6(a) shows that, during the first 30 min at 46°C, the amount of ^{125}I -EGF internalized by

heat-shocked A-431 cells is comparable with that of cells maintained at 37°C. At later times, however, the amount of ^{125}I -EGF internalized in heat-stressed cells is approx. 30% less than in cells incubated at 37°C.

The difference in ligand internalization seen after prolonged heat stress suggests that the intracellular processing of ligand-receptor complexes may be affected. At 37°C, internalized ^{125}I -EGF passes through intracellular compartments before reaching lysosomes, where it is degraded [9]. Evidence of this degradation is the detection of mono[^{125}I]iodotyrosine in the culture medium [9]. Dishes of A-431 cells, incubated with or without ^{125}I -EGF for 30 min at 37°C or 46°C, were washed four times with a pH 4.0 buffer to remove free and surface-bound growth factor. DMEM containing 0.1% BSA was added, and the cells were then

incubated for 90 min at 37 °C or 46 °C to allow degradation of the internalized ^{125}I -EGF. Conditioned medium was removed from the dishes and total ^{125}I radioactivity was measured. The medium was then precipitated with TCA, and non-TCA-precipitable radioactivity (^{125}I -EGF degradation products, i.e. mono[^{125}I]iodotyrosine) was determined [38]. Fig. 6(b) shows that 3-fold more total radioactivity, and 7-fold more non-TCA-precipitable radioactivity, were recovered in the medium from cells incubated at 37 °C than at 46 °C. Of the total radioactivity in the conditioned medium from cells incubated at 37 °C, 70 % was non-TCA-precipitable, indicating the presence of ^{125}I -EGF degradation products. In the medium from cells incubated at 46 °C, approx. 30 % of total radioactivity is non-TCA-precipitable.

Although a smaller amount of ^{125}I -EGF degradation products was found in the medium from heat-stressed cells, analysis of radioactivity from the intracellular compartments did not indicate a corresponding increase in intracellular degradation products (Fig. 6b). Therefore, the observed decrease in degradation products in the medium is not due to accumulation within the cells. Since cells maintained at either temperature internalized approximately the same amount of ^{125}I -EGF, these data suggest that some component(s) of the intracellular ligand-degradation process is inhibited, or at least significantly decreased, by heat stress.

DISCUSSION

Although a substantial amount of information exists on the regulation of gene expression and protein synthesis in cells subjected to heat shock, few studies have addressed the effects of heat stress on intrinsic signal-transduction pathways. Previous studies have suggested that some components of receptor-linked signal-transduction systems may remain intact during heat stress. Induction of heat shock in HA-1 and Balb 3T3 cells resulted in the rapid stimulation of phosphoinositide turnover and accumulation of InsP_3 [42]. Also, an increase in general protein tyrosine phosphorylation has been reported for several cell lines subjected to heat shock [43]. One recent report indicates that serine phosphorylation and activity of S6 protein kinase are stimulated by heat exposure [44]. These results suggest that receptor-linked signal-transduction pathways could be utilized in the heat-stress response.

Other work, however, indicates that peptide-hormone binding to cells is decreased by heat stress. One group [4] has reported that a 43 °C heat shock inhibits insulin binding to insulin receptors on HA-1 cells in a time-dependent manner. Similarly, another group [45] found that ^{125}I -EGF binding to Rat-1 fibroblasts decreased with increasing heat stress. However, we find that ^{125}I -EGF binding to A-431 cells is unaffected by heat stress for up to 90 min. There is a major difference among the protocols used that may explain the somewhat contrasting results. In the two previous reports [4,45], cells were subjected to different times of heat stress before being returned to 37 °C for the ligand-binding assays. Our ^{125}I -EGF binding assays were done entirely under heat-shock conditions. This includes direct addition of ^{125}I -EGF to cells at 46 °C, instead of shifting cells back to 37 °C before ^{125}I -EGF addition. We find that the EGF receptor also retains many of its properties associated with signal transduction during heat stress. Receptor autophosphorylation after EGF binding at 46 °C in both A-431 cells and human fibroblasts is similar to autophosphorylation achieved at 37 °C, indicating that EGF-receptor tyrosine kinase activity is not diminished by heat shock of whole cells. In contrast, when A-431 membrane preparations were heated to 45 °C, the EGF receptor kinase was

rapidly inactivated, though ^{125}I -EGF binding was unaffected [26].

A number of cytoplasmic changes are normally associated with the heat shock of cells. These include a rapid fall in intracellular pH [46], an increase in cytoplasmic Ca^{2+} [47] and a decrease in cellular ATP [48]. PLC- γ 1 is a high-stoichiometric substrate of the EGF receptor kinase and is mostly located in the cytoplasm. Upon EGF stimulation, however, a substantial fraction of PLC- γ 1 is rapidly translocated to the inner face of the plasma membrane [49]. One might expect the translocation and/or activation of PLC- γ 1 to be affected by the cytoplasmic changes associated with heat shock. One group has recently reported that another type of cellular stress, the addition of 6–300 mM-ethanol to rat hepatocytes, inhibits EGF-stimulated PLC activity [50]. They cite a decrease in the rate of EGF-induced increase in cytosolic free Ca^{2+} as evidence for diminished PLC activity.

We directly examined the levels of PLC- γ 1 phosphorylation and of inositol phosphate formation after EGF stimulation during heat shock. PLC- γ 1 is phosphorylated in response to EGF stimulation of heat-stressed human fibroblasts and A-431 cells, and the levels of phosphorylation are similar to those measured in cells incubated at 37 °C with EGF. Also, EGF stimulation of heat-treated A-431 cells results in a rapid increase in cellular inositol phosphates, the products of PLC- γ 1-mediated hydrolysis of PtdInsP_2 . The increases in inositol phosphates at 46 °C are nearly identical with those recorded after EGF addition at 37 °C. Therefore, heat-stressed A-431 cells show no difference from unstressed cells in the ability of this membrane-associated enzyme to hydrolyse PtdInsP_2 in response to EGF. Since the hydrolysis of PtdInsP_2 produces InsP_3 , which mediates subsequent changes in intracellular Ca^{2+} flux, it is relevant to address the issue of this pathway, or at least intracellular Ca^{2+} levels, in mediating heat-stress responses. PLC activity has been postulated to be involved in the increase in intracellular free Ca^{2+} associated with heat shock [42], and one group has recently demonstrated that free intracellular Ca^{2+} is needed for heat-shock factor binding to DNA, as a part of heat-stress gene induction [51]. The retention of EGF-mediated signal transduction in heat-stressed cells provides one possible mechanism for the mobilization of intracellular Ca^{2+} during the heat-shock response. However, there is no evidence that heat shock itself activates the EGF receptor. We did not observe increased levels of inositol phosphates in A-431 cells due to heat shock alone, and have only occasionally observed increased levels of tyrosine phosphorylation on the EGF receptor after heat shock in the absence of ligand.

In contrast with the receptor-linked pathway of PLC- γ phosphorylation and activation, the fate of ^{125}I -EGF-receptor complexes is affected by heat shock of A-431 cells. Others [52] have suggested that heat stress induces alterations in the lysosomal activity of malignant cells. Our results indicate that heat shock decreases the extent of degradation of internalized ^{125}I -EGF.

In view of the rapid inactivation of the EGF receptor at 45 °C *in vitro*, it is of interest to determine what component(s) of the intact cell are responsible for maintaining EGF receptor kinase activity at 46 °C and what role, if any, EGF-mediated signal transduction might have in the cellular heat-stress response.

The technical assistance of Deirdre Sanchez and Usha Barnela is gratefully acknowledged. We thank Susan Heaver for assistance in preparation of the manuscript, and Alexander Sorkin for helpful discussions. This research was supported by N.I.H. grant CA24071 and the Vanderbilt University Medical Scientist Training Program (S. M. L.).

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Received 28 January 1992; accepted 19 March 1992