Epidermal Growth Factor Disrupts Gap-Junctional Communication and Induces Phosphorylation of Connexin43 on Serine

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Growth factors regulate cellular proliferation and differentiation by activating plasma membrane tyrosine kinase receptors and triggering a cascade of events mediated by intracellular signaling proteins. The mechanism underlying growth factor modification of cellular functions, such as gap-junctional communication (gjc), has not been established clearly. Addition of epidermal growth factor (EGF) to T51B rat liver epithelial cells resulted in the rapid activation of EGF receptor tyrosine kinase activity followed by a transient dose-dependent disruption of gjc. This change did not result from the gross disturbance of membrane gap junction plaques as measured by immunofluorescence microscopy, but instead correlated with markedly elevated phosphorylation of the connexin43 (cx43) gap junction protein, a profound shift to predominantly phosphorylated forms of cx43, and the appearance of a novel phosphorylated cx43 protein. These changes in cx43 phosphorylation involved only serine residues. On restoration of gjc, these alterations in cx43 phosphorylation reverted to the pre-EGF treatment state. Both events were inhibited by the serine/threonine protein phosphatase inhibitor, okadaic acid. Therefore, unlike the case for pp60^{v-src}, EGF-induced disruption of gjc is not associated with tyrosine phosphorylation of cx43, but instead may result from phosphorylation of cx43 by activated intracellular signaling serine protein kinase(s).

INTRODUCTION

Gap junctions are aqueous channels formed in juxtaposed plasma membranes that permit the transfer of ions, nutrients, and other metabolites between adjacent cells (Loewenstein, 1981; Hertzberg and Johnson, 1988). They are ubiquitous structures found in virtually all animal cells, and they play important roles in normal organ function (Page and Manjunath, 1986; Garfield *et al.*, 1988; Hertzberg and Johnson, 1988), development (Caveney, 1985; Fraser *et al.*, 1987; Hertzberg and Johnson, 1988), and perhaps, growth control (Mehta *et al.*, 1986, 1991; Hertzberg and Johnson, 1988; Loewenstein, 1979; Yamasaki and Katoh, 1988; Zhu *et al.*, 1991).

Gap junctions are composed of hexamers of proteins termed connexins (Beyer *et al.*, 1988). The molecular mechanisms that regulate gap-junctional communication (gjc) are unclear; however, both up-regulation and down-regulation of gjc have been correlated with alterations in connexin protein phosphorylation (Loewenstein, 1984; Stagg and Fletcher, 1990; Laird *et al.*, 1991). Up-regulation of gjc has been associated with increased connexin32 (cx32) phosphorylation in liver hepatocytes treated with cyclic AMP (Saez *et al.*, 1986, 1990; Traub *et al.*, 1987) and increased cx43 phosphorylation in mouse S180 cells transfected with L-CAM DNA (Musil *et al.*, 1990b). Down-regulation of gjc has been observed recently in mouse primary keratinocytes, rat hepatocytes, WB cells, and Madin-Darby Canine Kidney cells treated with the tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA) or transformed by the *ras* oncogene (Takeda *et al.*, 1989; Bris-

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sette *et al.*, 1991; Oh *et al.*, 1991; Berthoud *et al.*, 1992). These divergent effects on gjc, which occur predominantly on serine, may be explained, in part, by different protein kinases phosphorylating unique serine and/or threonine sites. In contrast, disruption of gjc by the oncoprotein, pp60^{v-src}, has been associated with the phosphorylation of cx43 on tyrosine (Crow *et al.*, 1990, 1992; Filson *et al.*, 1990; Swenson *et al.*, 1990).

Growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor, bind to and activate their tyrosine kinase receptors (Ullrich and Schlessinger, 1990), setting into motion a variety of second messenger systems, which in turn activate a number of serine/threonine protein kinases (Ullrich and Schlessinger, 1990; Koch *et al.*, 1991; Sturgill and Wu, 1991). One result of receptor activation is the disruption of gjc (Maldonado *et al.*, 1988; Madhukar *et al.*, 1989); however, the mechanism(s) by which this occurs is unresolved. Because pp60^{v-src}'s effects on gap junctions is associated with tyrosine phosphorylation of cx43, we initiated a study to determine if a similar or different mechanism was involved for growth factor modulation of gjc.

METHODS

Measurement of gjc

gjc was measured in rat liver epithelial cells (clone T51B) by enumeration of the number of cells that become fluorescent after microinjection of the dye, Lucifer yellow, into a single cell as described by Crow *et al.* (1990). Briefly, cells grown to confluency in basal minimal essential medium-10% fetal calf serum (Boynton *et al.*, 1984) were treated with either phosphate-buffered saline (PBS) (control) or EGF (United States Biochemical) at various concentrations and for the times indicated. After the treatment period, cells were microinjected with a glass capillary needle prepared from a Flaming-Brown micropipette puller (Sutter Instrument, San Rafael, CA), containing 10% Lucifer yellow CH in 0.3 M lithium chloride. Individual cells were microinjected using a micromanipulator (Zeiss, Thornwood, NJ) and pneumatic injector (Eppendorf, Fremont, CA) and observed using a phase-contrast inverted microscope (Diaphot; Nikon, Garden City, NY) equipped with epifluorescence. Quantitation of gjc was performed 1–2 min after microinjection by counting the number of fluorescent cells.

Measurement of EGF Receptor Activation

Confluent T51B rat liver epithelial cells were treated with either PBS or EGF (50 ng/ml) for the indicated times. Immunoprecipitations and immunoblotting were performed as described previously (Crow *et al.*, 1990). Cells were lysed by the addition of RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecylsulfate, 10 mM Tris (hydroxymethyl) aminomethane [Tris], pH 7.2) and clarified in an ultracentrifuge (model TL-100; Beckman, Fullerton, CA) at 400 000 × g for 20 min at 4°C. Lysates were incubated with either rabbit nonimmune serum or EGF receptor antibody (a gift of Dr. F. Fazioli, National Institutes of Health, Bethesda, MD) for 1.5 h on ice. Immune complexes were collected by incubation with protein A-S. aureus (1:20 ratio of antisera) for 30 min and centrifugation at 1400 × g for 15 min at 4°C. Immune complexes were washed four times with RIPA buffer and extracted by boiling in sodium dodecyl sulfate (SDS)-polyacrylamide gel sample buffer for 3 min. Proteins

were resolved on an SDS-containing 7.5% polyacrylamide gel (Laemmli, 1970) and then electrotransferred to an Immobilon P membrane, (Millipore, Bedford, MA). Phosphorylated EGF receptor was detected by immunoblotting (Lau, 1986). The membrane was blocked with 5% bovine serum albumin in 10 mM Tris (pH 7.4)-0.15 M NaCl (TSB) for 1 h at room temperature (RT). It was incubated with 2 µg/ml phosphotyrosine monoclonal antibody (Upstate Biotechnology, Lake Placid, NY) for 4 h at RT on a rocker and then washed in TSB containing 0.05% Tween-20 for 30 min. Immune complexes were detected with [¹²⁵I]sheep anti-mouse IgG (1 × 10⁶ cpm) for 1 h at RT on a rocker, followed by washing as described above. The dried membrane was autoradiographed using X-Omat film (Eastman Kodak, Rochester, NY) with the aid of an intensifying screen.

Immunofluorescence Microscopy

T51B cells grown to confluency on glass coverslips were treated with PBS or EGF (50 ng/ml) for the indicated times. Cells were rinsed with PBS and fixed with 3% formaldehyde (wt/vol) in PBS for 20 min at RT. Cells were permeabilized with 0.2% Triton X-100 in PBS for 2 min and reduced with 26 mM sodium borohydride for 15 min at RT. After a 15 min wash in PBS, samples were incubated with either rabbit antiserum against cx43 CT368 peptide (residues 368–382) or preimmune serum at 1:1000 dilutions for 2 h at RT. Samples were washed in PBS and blocked with normal goat serum (1:50 dilution) for 30 min at RT. The samples were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:80 dilution) for 1 h at RT, washed in PBS, and mounted with 50% PBS/50% glycerol containing 1 mg/ml *p*-phenylenediamine (Sigma, St. Louis, MO). Fluorescent and phase contrast images were visualized and photographed using a Zeiss Axioplan Universal microscope equipped with epifluorescence.

Radiolabeling, Immunoprecipitation, and Quantitation of cx43

Confluent T51B cells were radiolabeled with either [³⁵S]methionine (NEG-072; New England Nuclear, Boston, MA) at 50 µCi/ml in methionine-free medium or [³²P]_i (NEX-053; New England Nuclear) at 0.5 mCi/ml in phosphate-deficient medium for 3 h at 37°C. Cells were treated with either PBS or EGF at 20 ng/ml PBS for various times. Termination of growth factor treatment coincided with the end of the 3-h radiolabeling period. Dose-response experiments of cx43 phosphorylation were performed using cells treated with either PBS or EGF at various concentrations for 30 min. Cells were washed twice with PBS on ice and either used immediately or frozen on the dish at −70°C. Cells were lysed in RIPA buffer, clarified, and immunoprecipitated with either normal rabbit serum or antiserum against the CT368 peptide of cx43. Immunoprecipitated proteins were resolved on SDS-containing 7.5–15% gradient polyacrylamide gels. Autoradiograms of the fluorographed gel were prepared using Kodak X-Omat film for 4–6 d at −70°C without the aid of an intensifying screen.

Gel pieces containing [³⁵S]- or [³²P]-labeled cx43 proteins were excised, rehydrated, and counted in 7.5% Scintigest (New England Nuclear)-Scintiverse fluid (Fisher Scientific, Pittsburgh, PA) using a Beckman LS5000 counter. The radioactivity of all gel bands was corrected for background gel lane radioactivity.

Phosphoamino Acid Analysis

[³²P]-labeled cx43₂ was immunoprecipitated and gel-purified, as described previously, from T51B cells after PBS or EGF (20 ng/ml) treatment for the indicated times. cx43₁ and cx43₃ were also subjected to phosphoamino acid analyses. As a control for the presence of phosphotyrosine, cx43₂ from *v-src*-transformed Rat-1 fibroblasts was analyzed. Acid-hydrolysates of [³²P]proteins bound to Immobilon-P membranes were resolved on thin-layer cellulose plates in two dimensions at pH 1.9 and 3.5, as previously described (Kamps and

Sefton, 1989; Crow *et al.*, 1990). Autoradiography was performed using Kodak X-Omat film with the aid of intensifying screens at -70°C for 14–21 d.

Okadaic Acid Experiments

T51B cells were mock-treated with PBS and/or dimethyl formamide (DMF) or treated with EGF (25 ng/ml PBS) for 30 min or 3 h in the presence or absence of 1 nM okadaic acid (OA) in DMF. For the 30-min treatment time, OA was added 1.5 h before the addition of EGF; for the 3-h treatment time, OA was added 1 h after EGF addition. At the end of the EGF treatment periods, all cultures were assayed for levels of gjc by microinjection or cx43 phosphorylation by immunoprecipitation and SDS-polyacrylamide gel analysis (see above). Attempts to examine gjc and cx43 phosphorylation at higher OA concentrations for prolonged time periods were confounded by alterations of cellular morphology and organization.

RESULTS

EGF Induces a Rapid Transient Inhibition of gjc

Addition of EGF (25 ng/ml) to T51B epithelial cells resulted in a rapid reduction in gjc (from 60 cells in control cultures to 40 cells), which was detectable at 10 min, the earliest time point examined (Figure 1A). This effect was time dependent with further reductions in gjc occurring at 20 min (to ~ 10 cells) and at 60 min, where the lowest level was reached (< 2 cells contained fluorescent dye). This was a transient effect of EGF in that gjc returned nearly to control values (~ 50 cells) at 2 h and remained elevated through 24 h (Figure 1B). Mock-treated cells showed only a slight decline in gjc over the same time course (Figure 1A). A similarly rapid, though less extensive, decline in gjc after EGF treatment of normal rat kidney (NRK) or Balb/C 3T3 cells was reported by Maldonado *et al.* (1988).

EGF Receptor is Rapidly Activated

EGF treatment of T51B cells for 2 min induced a rapid appearance of phosphotyrosine (Ptyr) in a protein of ~ 180 kDa (Figure 1C). Its Ptyr level achieved a maximum level at 5 min and then progressively declined nearly to control levels by 1 h. The protein's apparent mass, specific immunoreactivity to EGF receptor antibody, and EGF-inducible Ptyr content are consistent with it being the EGF receptor. This rapid activation of the EGF receptor appeared to occur well before the disruption of gjc, suggesting a causal link between activation of receptor kinase activity and disruption of gjc. The rapid loss of EGF receptor Ptyr observed here is consistent temporally with the reported desensitization of the receptor tyrosine kinase activity that is associated with the phosphorylation of serine residues at positions 1046 and 1047 of the receptor (Countaway *et al.*, 1992).

EGF Disrupts gjc Without a Gross Disturbance of Gap Junction Plaques

To examine the possibility that EGF disruption of gjc might be due to a disturbance of membrane gap junction

Figure 1. EGF modulation of gap-junctional communication and activation of EGF receptor in rat liver epithelial cells. Cells were treated with EGF for up to 60 min (A) or 24 h (B) and the changes in gap-junctional communication measured at various times as described in METHODS. These data represent the averages ($\pm$ SE) of 6–10 determinations (up to 60 min) or 2–6 determinations (up to 24 h). (C) To measure the time course of activation of the EGF receptor tyrosine kinase activity, T51B cells were treated with EGF (50 ng/ml) for the indicated times, lysed, and immunoprecipitated with either nonimmune serum (NI) or EGF receptor antibody (E). Activated EGF receptor was detected by immunoblotting with phosphotyrosine (Ptyr) antiserum as described in METHODS.

plaques, T51B cells were analyzed by immunofluorescence microscopy using rabbit antiserum directed against the C-terminal peptide of cx43 (Beyer *et al.*, 1987; Crow *et al.*, 1990). Discrete punctate cx43 antibody reactions were detected in the periphery of mock-treated cells with little reaction in the cytoplasm, except for a single diffuse juxtanuclear reaction (Figure 2). The peripheral reactions probably represent antibody binding to cx43 in plasma membrane gap junction plaques, as previously noted (Crow *et al.*, 1990; Musil and Goodenough, 1991). The juxtanuclear reactions appear similar to those observed in Golgi structures by Musil and Goodenough (1991). EGF treatment of these cells for 10 or 30 min (when gjc is markedly diminished) did not result in a noticeable alteration in cx43 antibody reactions in either the plasma membrane or juxtanuclear regions. After 3 h of EGF treatment, the plasma membranes of T51B cells appeared to contain a comparable number of punctate reactions; however, they were smaller in size (Figure 2). The functional significance of this difference is uncertain, as gjc was nearly restored to control levels at this time (see Figure 1B). Both plasma membrane and juxtanuclear reactions appeared to be specific because preimmune serum did not produce

Figure 2. Subcellular localization of cx43 in EGF-treated T51B rat liver epithelial cells. Cells were treated with EGF (25 ng/ml) for 10 min, 30 min, and 3 h, permeabilized, then reacted with either the cx43 CT368 peptide antiserum or preimmune serum. Primary immune complexes were detected by their reaction to FITC-conjugated secondary antiserum. Fluorescent and the corresponding phase contrast images are presented.

analogous results in mock or EGF-treated cells (only data for 30 min EGF treatment are shown; Figure 2). Although these data do not exclude the possible existence of finer alterations in gap junction structure, they

do suggest that the striking EGF-induced reductions in gjc are unlikely to be the result of gross alterations in gap junction plaque organization.

EGF Stimulates a Shift in Pattern of cx43 Proteins

Since the EGF receptor is a tyrosine protein kinase, we investigated the possibility that the EGF disruption of gjc was associated with altered phosphorylation of cx43 and, if so, whether or not tyrosine phosphorylation was involved, as previously reported for the nonreceptor tyrosine kinase, pp60^{v-src} (Crow *et al.*, 1990, 1992; Filson *et al.*, 1990; Swenson *et al.*, 1990). cx43 was studied because Northern blotting experiments showed that, of the connexin cDNAs tested (cx46, cx43, cx40, cx37, cx32, and cx26), only cx43 RNA was expressed in T51B cells (Goldberg and Lau, unpublished observations).

Mock-treated cells produced a [³⁵S]cx43 pattern consisting of a predominant 43 kDa (cx43_{np}) and minor 45 (cx43₁) and 47 (cx43₂) kDa proteins (Figure 3A, lane 2). An identical cx43 pattern was observed previously in fibroblasts (Crow *et al.*, 1990). EGF treatment for 10 and 30 min resulted in a striking alteration in the proportions of the different cx43 forms. [³⁵S]labeled cx43₂ became the major form, and, in addition, a faint but novel [³⁵S]protein of >47 kDa (cx43₃) appeared (Figure 3A, lanes 6 and 10). At the same time, [³⁵S]cx43_{np} decreased markedly, whereas cx43₁ appeared to decrease slightly. After 3 h of EGF treatment, the [³⁵S]cx43 protein pattern appeared very similar to that observed in the mock-treated cells (Figure 3A, compare lanes 14 and 2). These proteins were specifically immunoprecipitated by the cx43 CT368 antiserum because they were not recognized by nonimmune serum (Figure 3A, lanes 1, 5, 9, and 13).

To provide quantitative assessments of these changes, the [³⁵S]radioactivity of each different cx43 protein band was determined by liquid scintillation counting, and its proportion of the total cx43 radioactivity was calculated. This analysis revealed that the percentage of phosphorylated cx43₂ increased from 13% of the total [³⁵S]cx43 in mock-treated cells to 39 and 36% in 10 and 30 min EGF-treated cells, respectively (Figure 4A). Similarly, cx43₃ went from an undetectable level in mock-treated cells to 10 and 13% at 10 and 30 min of EGF-treatment, respectively. After 3 h of EGF-treatment, the percentages of cx43₂ and cx43₃ decreased toward control values reaching 17 and 4%, respectively. In contrast, percentages of cx43_{np} and cx43₁ decreased on EGF treatment (Figure 4A). For example, about one-half of cx43_{np} was lost at the 10- and 30-min EGF-treatment time points (59 to 30 and 32%, respectively). After 3 h of EGF-treatment, the percentages of both proteins increased and returned almost to the mock-treated cell levels.

Figure 3. Phosphorylation of cx43 in EGF-treated T51B rat liver epithelial cells. (A) Cells were either mock-treated or treated with EGF for the indicated times and labeled with [^{35}S]methionine or $^{32}\text{P}_i$. Cx43 was immunoprecipitated from cell lysates with either nonimmune rabbit serum (lanes 1, 4, 5, 8, 9, 12, 13, and 16) or anti-cx43 CT368 peptide serum (lanes 2, 3, 6, 7, 10, 11, 14, and 15) and resolved on SDS-polyacrylamide gels, as described in METHODS. (B) A shorter autoradiographic exposure (2 d) of the same gel in A to show the cx43₂ and cx43₃ phosphorylation after 10 and 30 min EGF-treatment more clearly (lanes 7 and 11). (C) Phosphoamino acid analysis of cx43₂ from T51B cells either mock-treated or treated with EGF for 5, 10, 30, and 180 min. Results from analysis of cx43₂ from v-src-transformed Rat-fibroblasts are also presented. Positions of the ninhydrin-stained unlabeled phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) standards are outlined. Direction of migration for the first dimension (pH 1.9) and the second dimension (pH 3.5) are indicated at the bottom right corner. Analyses of cx43₁ and cx43₃ demonstrated the presence of only phosphoserine.

EGF Stimulates a Marked Increase in cx43 Phosphorylation

In [^{32}P]-labeled mock-treated T51B cells, cx43₁ and cx43₂ were phosphorylated (Figure 3A, lane 3). These proteins are analogous to the fibroblast connexin 45- and 47-kDa proteins that represent phosphorylated forms of the nonphosphorylated cx43_{np} protein (Crow *et al.*, 1990). EGF treatment for 10 and 30 min stimulated large increases in phosphorylation of cx43 that coincided with the disruption of gjc (Figure 3A, lanes 7 and 11). This effect was limited mostly to cx43₂ and the novel cx43₃ protein (Figure 3B, lanes 7 and 11, represents a shorter exposure of gel proteins shown in Figure 3A). After 3 h of EGF exposure, when gjc had returned nearly to normal levels, the enhanced phosphorylation of cx43 proteins diminished to control levels with cx43₁ and cx43₂, again representing the major phosphorylated species (Figure 3A, lane 15).

Direct measurement of the [^{32}P]cx43 radioactivity provided quantitative estimates of the large increases in cx43 phosphorylation observed by autoradiography. After 10 and 30 min of EGF-treatment, the [^{32}P]radioactivity of cx43₂ increased approximately eight- and sixfold, respectively, when normalized to the corresponding mock-treatment value (Figure 4B). These increments contrasted sharply with phosphorylation of cx43₁, which increased less than twofold at the same time periods. The increase in [^{32}P]radioactivity of cx43₂ was not explained solely by the increase (about threefold) in its percentage of total [^{35}S]cx43 (Figure 4A). Be-

cause cx43₃ was undetectable in mock-treated cells, it was not possible to calculate similar relative increases in its phosphorylation. However, its absolute [^{32}P]radioactivity increased from an undetectable level in mock-treated cells to average values of 164 cpm at 10 min and 659 cpm at 30 min of EGF-treatment (Figure 4B). At 180 min of EGF-treatment, cx43₃ [^{32}P]radioactivity decreased to an average level of 47 cpm.

EGF-Induced Phosphorylation of cx43 Occurs on Serine

Phosphoamino acid analysis of the cx43 proteins showed that phosphoserine (Pser) was the predominant phosphoamino acid detected in cx43₂ isolated from both mock- and EGF-treated T51B cells (Figure 3C). Interestingly, this protein from EGF-treated cells clearly lacked P_{tyr}, in contrast to phosphorylated cx43₂ isolated from v-src-transformed fibroblasts (Figure 3C). Phosphoamino acid analyses of all other phosphorylated cx43 proteins (cx43₁ and cx43₃) yielded identical results.

EGF's Effects are Concentration Dependent and Occur in a Concomitant Fashion

These effects of EGF were concentration dependent, with the lowest tested dose of 0.5 ng/ml resulting in an ~50% decrease in gjc and a concomitant nearly twofold rise in cx43 phosphorylation (Figure 5). Higher concentrations of EGF up to 10 ng/ml resulted in further

increases in cx43 phosphorylation and depression of gjc. This effect on gjc was observed at EGF concentrations similar to those used in previous studies (Maldonado *et al.*, 1988; Madhukar *et al.*, 1989).

OA Inhibits Dephosphorylation of cx43 and Reestablishment of gjc

To provide more evidence that the phosphorylation of cx43 induced by EGF may be directly involved in the alteration of gjc, the effects of the serine/threonine protein phosphatase inhibitor, OA (Cohen *et al.*, 1990), were examined. Cells treated with EGF for 3 h were exposed to OA commencing 1 h after EGF addition, which corresponded to the period when cx43 is undergoing dephosphorylation and gjc is becoming reestablished (see Figures 1, A and B and 3A).

OA limited the restoration of gjc to approximately one-third of the level that is usually observed after 3 h EGF treatment (Figure 6). This maintenance of depressed gjc was accompanied by the continued elevated phosphorylation of the cx43₂ and cx43₃ proteins by OA (Figure 6, compare lanes 10 and 12). OA also decreased gjc slightly in mock-treated or 30 min EGF-treated cells, which was accompanied by an increase in or maintenance of the cx43₂ and cx43₃ phosphorylation, respectively (Figure 6, compare lanes 4 and 8).

DISCUSSION

The data presented here suggest the possibility that EGF's transient disruption of gjc in T51B rat liver epi-

Figure 4. Quantitation of EGF-induced alterations in the species and phosphorylation of cx43. (A) EGF-induced changes in the proportions of cx43 proteins from EGF-treated T51B cells. The radioactivity of gel bands containing [³⁵S]-labeled cx43_{np}, cx43₁, cx43₂, and cx43₃ was determined by liquid scintillation spectrometry as described in METHODS. ND in the top part of the chart signifies that cx43₃ was not detected by liquid scintillation counting or autoradiography in mock-treated T51B cells. The percentage of the total [³⁵S]radioactivity (100% values averaged between 978 and 2030 cpm) that each cx43 protein represented was determined for the various mock- and EGF-treatment times. These data represent the averages ($\pm$ SE) of three independent determinations. (B) Increases in phosphorylation of cx43 proteins from EGF-treated T51B cells. [³²P]-labeled protein gel bands corresponding to cx43₁ and cx43₂ from experiments such as shown in Figure 3A were excised and their radioactivity determined by liquid scintillation counting, as described in METHODS. Increases in the [³²P]radioactivity of cx43₁ and cx43₂ proteins from EGF-treated cells were normalized to the corresponding mock-treatment value and the resulting ratios charted. The ND indication for cx43₃ of mock-treated cells signifies that it was not detected (as in A). Thus, data for cx43₃ represent the absolute [³²P]radioactivity measured by liquid scintillation counting, because a ratio could not be calculated. Results represent averages ($\pm$ SE) of two separate experiments, except for the 30 min data that were calculated from data of seven experiments.

Figure 5. Concentration dependence of EGF's effects on gap-junctional communication and cx43 phosphorylation. Cells were treated with EGF at the indicated concentrations for 30 min and then assayed for their extent of gap-junctional communication as described in METHODS. [32 P]-labeled companion cultures were harvested, lysed, and cx43 immunoprecipitated and resolved by SDS-polyacrylamide gel electrophoresis. The radioactivity of the cx43 band region was determined by liquid scintillation counting. Increases in cx43 phosphorylation were expressed relative to the average control value of 129 cpm. The data represent averages ($\pm$ SE) of between two and four independent determinations.

thelial cells may involve altered cx43 phosphorylation. Loss of gjc correlated with an equally rapid marked increase in phosphorylation and proportion of cx43₂ and the transient appearance of a novel phosphorylated cx43₃ protein. Furthermore, these changes in cx43 phosphorylation reverted nearly to control levels on resumption of gjc by the T51B cells. These effects were also clearly dose dependent where the lowest EGF concentration of 0.5 ng/ml produced concomitant effects on gjc and cx43 phosphorylation. OA's effective inhibition of cx43 dephosphorylation was matched by its ability to limit the reestablishment of gjc that is typically observed 3 h after EGF addition. Finally, EGF's disruption of gjc did not appear to result from gross disturbances of gap junction plaque organization that were observable at the light microscopic level.

We and others have reported previously the existence of slower migrating cx43 proteins in various cell types that resulted from serine phosphorylation of nonphosphorylated cx43_{np} (Crow *et al.*, 1990; Musil *et al.*, 1990a,b, 1991; Kadle *et al.*, 1991; Laird *et al.*, 1991; Berthoud, 1992). It is likely that serine phosphorylation is also responsible for producing the phosphorylated cx43₁₋₃ proteins described in this study. Our cx43_{np}, cx43₁, and cx43₂ proteins appear very similar to the CX43-NP, CX43-P₁, and CX43-P₂ proteins, respectively, described by Musil and Goodenough (1991).

The EGF-induced increase in the stoichiometry of cx43₂ and cx43₃ phosphorylation resulted in these proteins amounting to nearly 50% of the total cx43 in 10-

and 30-min EGF-treated cells, compared with only 13% in the mock-treated controls. Thus, an average of three out of six cx43 molecules in a gap junction hemichannel might be one of these two phosphorylated forms. However, it is uncertain whether or not EGF disruption of gjc requires elevated amounts and phosphorylation of cx43₂ and/or the presence of the novel cx43₃ protein.

The apparent dephosphorylation of the cx43₂ and cx43₃ proteins, which coincided with the reestablishment of gjc, initially suggested the rapid action of an intracellular membrane-associated, Pser phosphatase(s). The ability of nanomolar concentrations of OA to effectively block cx43 dephosphorylation and the reestablishment of gjc supports this suggestion and implicates serine/threonine protein phosphatase 2A and/or 3 in this process (Cohen *et al.*, 1990; Honkanen *et al.*,

Figure 6. Effects of okadaic acid on EGF-induced alterations in gap-junctional communication and cx43 phosphorylation. Cells were mock-treated or treated with EGF (25 ng/ml) for 30 min or 3 h in the presence or absence of the phosphatase inhibitor, OA (1 nM). The level of gjc in these cells was determined as described in METHODS and Figure 1. An autoradiogram of a polyacrylamide gel containing [32 P]cx43 immunoprecipitated from metabolically labeled companion cells is shown in the top portion of the Figure. [32 P]cx43 proteins from cells treated with the following: lanes 1 and 2, control; lanes 3 and 4, OA; lanes 5 and 6, EGF for 30 min; lanes 7 and 8, EGF for 30 min plus OA; lanes 9 and 10, EGF for 3 h; and lanes 11 and 12, EGF for 3 h plus OA. Odd numbered lanes, nonimmune rabbit serum immunoprecipitates; even numbered lanes, anti-cx43 CT368 peptide serum immunoprecipitates. The cx43_{2,3} at the top left margin indicates that the cx43₂ and cx43₃ proteins were not resolved well from one another on this particular gel due to interference by the immunoglobulin heavy chain migrating immediately above the cx43 region.

1991). Although protein phosphatase 1 is also inhibited by OA, it requires micromolar concentrations to do so (Cohen *et al.*, 1990), and thus it is probably not involved in the OA effects reported here.

Maldonado *et al.* (1988) suggested two possible mechanisms for growth factor reduction in gjc: direct phosphorylation of the channel protein on tyrosine by the receptor kinases or one involving an increase in intracellular Ca^{2+} . It is clear from our data that, unlike pp60^{v-src}'s disruption of gjc that is associated with phosphorylation of cx43 on tyrosine (Crow *et al.*, 1990; Filson *et al.*, 1990; Swenson *et al.*, 1990), the activated EGF receptor does not phosphorylate cx43 on tyrosine in T51B cells directly or indirectly (by activation of another intracellular tyrosine kinase) but rather induces serine phosphorylation by an unknown protein kinase. These results suggest that the transmembrane EGF receptor tyrosine kinase and intracellular tyrosine kinases, exemplified by pp60^{v-src}, may operate through two distinct molecular pathways to disrupt gjc. The second mechanism suggested by Maldonado *et al.* (1988), involving Ca^{2+} , is also unlikely because, although EGF increases T51B intracellular Ca^{2+} by an extracellular Ca^{2+} -dependent means (Hill *et al.*, 1988), disruption of gjc is independent of extracellular Ca^{2+} in T51B cells (Danesh and Boynton, unpublished observations). Furthermore, increasing intracellular Ca^{2+} by using agonists such as ionomycin or thrombin does not interrupt gjc in T51B cells (Danesh and Boynton, unpublished observations).

The activated EGF receptor interacts with, and phosphorylates, several substrates with known biochemical functions, such as phospholipase C- γ , *ras* GTPase-activating protein, and numerous protein kinases (Carpenter and Cohen, 1990). EGF's disruption of gjc and stimulation of cx43 serine phosphorylation in T51B cells suggests the involvement of a serine kinase that transduces signals from the activated EGF receptor intracellularly. One possible candidate might be protein kinase C whose activation by TPA is associated with disruption of gjc in a manner similar to EGF (Brissette *et al.*, 1991; Oh *et al.*, 1991; Berthoud *et al.*, 1992). The demonstration that the *ras* oncoprotein disrupts gjc and induces cx43 serine phosphorylation also suggests the possibility that EGF may down-regulate gjc through *ras*-dependent events (Brissette *et al.*, 1991). The serine protein kinase that might mediate this *ras* effect is unclear, but again, protein kinase C is a possible candidate. However, nerve growth factor and TPA modulation of the growth and differentiation of PC12 cells has been shown recently to be dependent on a *ras*-mediated activation of mitogen-activated protein (MAP), ribosomal S6, and raf-1 kinases (Thomas *et al.*, 1992; Wood *et al.*, 1992).

EGF stimulates a cascade of serine/threonine protein kinase activities (Carpenter and Cohen, 1990; Ahn *et al.*, 1990a,b, 1991). One of the better characterized of these is MAP-2 kinase (Ahn *et al.*, 1991; Rossomando

et al., 1989). Our computer-assisted analyses have identified numerous protein kinase phosphorylation consensus sequences in rat heart cx43, including two putative MAP-2 kinase sites (pro-leu-ser-pro) (Alvaréz *et al.*, 1991) located at residues 252–255 and 276–279. In addition, protein kinase C, p34^{cdc2}, casein kinase I, and glycogen synthase kinase-3 sites (Kennelly and Krebs, 1991), where the last of these may be phosphorylated in a "hierarchical" manner with adjacent MAP-2 kinase sites, were noted (Kanemitsu, Kurata, Cooper, and Lau, unpublished observations). Whether or not these cx43 putative consensus sites are actually phosphorylated under physiological conditions remains to be determined. Nevertheless, the observations presented in this report suggest that regulation of gap junctions may involve, in part, multisite phosphorylation of cx43, orchestrated by a complex interplay of intracellular signaling protein kinases.

Hypothetically, disruption of gjc in epithelial cells by EGF may interrupt the intercellular passage of cellular regulatory molecules, which may be involved in mediating cell growth or differentiation. The precise nature of these regulatory molecules is unclear at the present time. It has been proposed that the molecular mechanisms of growth inhibition in normal cells may involve several processes: interruption of cell cycle progression, stimulation of terminal differentiation, senescence, or apoptosis (Weinberg, 1991). The ability of growth factors to provoke cell growth and/or differentiation may involve not only the stimulation of positive growth regulatory pathways (involving proto-oncogene proteins), but perhaps also the simultaneous blockade of the cell's growth inhibitory pathways (involving tumor suppressor gene proteins) (Weinberg, 1991). Oncogenes that disrupt gjc, such as *src*, *yes*, *fps*, and *ras* (Crow *et al.*, 1990, 1992; Filson *et al.*, 1990; Swenson *et al.*, 1990; Brissette *et al.*, 1991; Kurata and Lau, unpublished observations), may permanently stimulate cell growth and induce the transformed phenotype by participating directly in growth stimulatory pathways, in addition to interfering with components of the growth inhibitory pathway. In this specific context, connexin genes and proteins might be viewed as components of this intracellular growth inhibitory pathway (Lee *et al.*, 1991; Mehta *et al.*, 1991).

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