A Negative Feedback Loop Attenuates EGF-induced Morphological Changes

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Abstract. Activation of the EGF receptor tyrosine kinase by ligand indirectly activates a series of other cellular enzymes, including protein kinase C. To test the hypothesis that phosphorylation of the EGF receptor by protein kinase C provides an intracellular negative feedback loop to attenuate EGF receptor signaling, we used scanning EM to follow the characteristic EGF-induced retraction of lamellipodia and concomitant cell shape changes. Wild type and mutant EGF receptors were expressed in receptor-deficient NR6 cells. The mutant receptors were prepared by truncation at C′ terminal residue 973 (c'973) to provide resistance to ligand-induced down regulation that strongly attenuates receptor signaling and by replacement of threonine 654 (T^{654}) with alanine (A^{654}) to remove the site of phosphorylation by protein kinase C. Cells expressing WT and c'973 EGF receptors demonstrated characteristic lamellipodial retraction after exposure to EGF, with the non-down regulating c'973 EGF receptors responding more rapidly. Exposure of cells to TPA blocked this response. Replacement of T^{654} by alanine resulted in EGF receptors that were resistant to TPA. Cells expressing the A^{654} mutation underwent more rapid and more extensive morphologic changes than cells with the corresponding T^{654} EGF receptor. In cells expressing T^{654} EGF receptors, down regulation of protein kinase C resulted in more rapid and extensive EGF-induced changes similar to those seen in cells expressing A^{654} EGF receptors. These data indicate that activation of protein kinase C and subsequent phosphorylation of the EGF receptor at T^{654} lead to rapid physiological attenuation of EGF receptor signaling.

A complex network of growth stimulatory and growth inhibitory factors provides environmental information that regulates cell proliferation. Sufficient signal strength is required to elicit cell proliferation while mechanisms for attenuating signaling are necessary to limit inappropriate cell division. Posttranscriptional mechanisms that attenuate signaling by ligand-activated EGF receptors include down regulation and transmodulation. On ligand binding, EGF receptors, which are diffusely distributed on the cell surface, cluster in coated pits (Haigler et al., 1978), undergo internalization via a high affinity saturable endocytic process (Lund et al., 1990), and are ultimately degraded (Stoscheck and Carpenter, 1984). Mutant EGF receptors that fail to down regulate in response to ligand activation display increased mitogenesis and transformation at low concentrations of EGF (Chen et al., 1989; Wells et al., 1990), emphasizing the consequences of loss of this attenuation mechanism.

Other signaling pathways can decrease the ability of EGF to act. The most extensively characterized mechanism for transmodulation of the EGF receptor involves protein kinase C. Activated protein kinase C catalyzes phosphorylation of the EGF receptor at threonine 654 (T^{654})[1] (Hunter et al., 1984), resulting in decreased affinity of the receptor for EGF (Shoyab et al., 1979; Lin et al., 1986), decreased EGF-stimulated protein tyrosine kinase activity (Cochet et al., 1984; Friedman et al., 1984; Downward et al., 1985), and inhibition of ligand-induced receptor internalization and down regulation (Lund et al., 1990). Ligand activation of EGF receptors also results in phosphorylation at T^{654} (Whiteley and Glaser, 1986; Decker et al., 1990). This presumably results from EGF receptors activating phospholipase C (Hepler et al., 1987) and phospholipase D (Besterman et al., 1986; Wright et al., 1990) to generate diacylglycerol (DAG), the endogenous activator of protein kinase C (Nishizuka, 1984). It has been postulated that a second EGF-dependent attenuation mechanism exists wherein activation of the EGF receptor results in subsequent activation of protein kinase C and negative feedback attenuation occurs via receptor phosphorylation at T^{654}. Attenuation mechanisms different from down regulation may be especially important under certain conditions. For example, the activating ligands EGF, TGF-α, and myxovirus growth factors are synthesized as membrane precursors (Scott et al., 1983) and are capable of acting at the cell surface (Brachmann et al., 1989; Wong et al., 1989; Anklesaria et al.,

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1. *Abbreviations used in this paper*: A^{654}, alanine 654; DAG, diacylglycerol; SEM, scanning EM; T^{654}, threonine 654; PDGF, platelet-derived growth factor; TPA, 12-o-tetradecanoylphorbol-13-acetate.

1990) under conditions where receptor internalization does not occur.

It has been difficult to directly examine the potential role of protein kinase C in attenuating EGF-induced receptor responses. Ligand-induced receptor internalization and down regulation have complicated attempts to assess other attenuation mechanisms that may be operating. Down regulation occurs rapidly and is a prolonged process, whereas rapid recovery from protein kinase C–mediated inhibition occurs because DAG is rapidly metabolized (Bishop and Bell, 1986), protein kinase C is down regulated (Collins and Rozengurt, 1984; Ballester and Rosen, 1985) and phosphatases are activated (Cohen, 1989). By using mutant EGF receptors that are defective in ligand-induced internalization and down regulation, it has become possible to assess the existence of a protein kinase C–mediated negative feedback loop initiated by ligand-activated EGF receptors. With scanning EM (SEM), we have assessed cell morphology which is a sensitive assay of integrated biological responses elicited by EGF (Chinkers et al., 1981; Miyata et al., 1989). In response to EGF, membrane ruffling, lamellipodial retraction, and "rounding up" occur; these cytoskeletal changes are rapidly induced and involve disengagement of lamellipodia from the underlying substrate. Actin and α-actinin are redistributed in cells exposed to EGF (Schlessinger and Geiger, 1981); myosin, spectrin, ezrin, and fimbrin also show a reorganization coincident with these shape changes (Bretscher, 1989). Filopodia become more apparent during lamellipodial retraction as attachment to the substrate is lost. Eventually, the filopodia are subsumed as the cell becomes increasingly rounded with blebs and ridges becoming the most apparent features (Jackowski et al., 1990). We used morphological changes in NR6 cell lines expressing wild type and mutant EGF receptors that contain alanine at residue 654 (A^{654}) to remove the site of protein kinase C phosphorylation (Lin et al., 1986; Davis, 1988) and mutant EGF receptors defective in ligand-induced internalization and down regulation (c'973) (Chen et al., 1989; Wells et al., 1990) to critically examine the operation of this proposed negative feedback pathway of signal attenuation in vivo.

Materials and Methods

EGF Receptor Constructions

Three independently derived cDNA clones of the human EGF receptor were analyzed in parallel. Two clones, Gen (Ullrich et al., 1984) and UCSD (Lin et al., 1984), were isolated from A431 cells that have a 25-fold amplified EGF receptor gene. These were compared to a human placental isolate (A. W. and J. M. Bishop, unpublished data). Sequences covering the entire coding region from nucleotide 167 to 3880 (numbered according to Ullrich et al., 1984) were obtained using the dideoxynucleotide chain-terminating reaction (Sanger et al., 1977). Reactions and gels were run in parallel with primary sequences deduced at points of divergence. Results are shown in Table I. Only three differences were found; all occurred in the third base position of codons and did not alter the predicted amino acid sequence. This allowed use of mutants in the UCSD A431-derived clone without the possibility of an A431-specific mutation contributing to the responses that were observed.

Site-directed mutagenesis was used to produce various EGF receptor mutants. First, the threonine at amino acid 654 was replaced by an alanine residue (Lin et al., 1986); second, the EGF receptor was truncated at amino acid 973 (c'973) by the incorporation of an amber codon followed by a HindIII site (Chen et al., 1989), yielding a receptor that fails to undergo ligand-induced internalization and down regulation. The A^{654} variant of the c'973 mutant was constructed by replacing the C' terminus from the BglII site at nucleotide 2951 of the WT A^{654} mutant with the corresponding fragment of the c'973 mutant. All constructions were resequenced in the regions of mutation and in the juxtamembrane region.

Generation of EGF Receptor–Expressing Cells

The EGF receptor constructions were cloned into a Moloney murine leukemia virus–derived mammalian expression vector in the *gag* position (Wells and Bishop, 1988). This vector contains the neomycin–phosphotransferase gene in the *env* position. The constructions were introduced into NR6 cells, a 3T3 derivative that lacks endogenous EGF receptors (Pruss and Herschman, 1977), by the lipofectin reagent (Bethesda Research Laboratories, Bethesda, MD) and by retroviral infection. Cells were maintained at 37°C, 8% CO_2, and >60% humidity in MEMα supplemented with 10% FBS and 10 mM Hepes (pH 7.4). Stable, polyclonal lines consisting of more than 20 colonies each were established and maintained in complete media supplemented with the neomycin analog G418 (400 μg/ml, Gibco Laboratories, Grand Island, NY). Clonal lines were obtained by subcloning from these lines at limiting dilution. Expression of EGF receptors was assayed by specific binding of ^{125}I-EGF to the cells (Chen et al., 1989). Functioning of the receptors was assessed by ligand-induced tyrosine kinase activation measured using Western blotting of whole cell extracts with ^{125}I-labeled monoclonal anti-phosphotyrosine antibody PY-20 (Glenney et al., 1988), EGF-dependent incorporation of ^{3}H-thymidine into DNA, and cell growth. Two series of lines were isolated, one expressing ~100,000 ligand-binding sites per cell and a second expressing ~15,000 ligand-binding sites per cell. In all experiments the cells expressing either high or low numbers of receptors behaved identically.

Analysis of EGF Receptor Distribution by Fluorescence-activated Cell Scanning and Immunocytochemistry

For fluorescence-activated cell scanning analysis, cells were dissociated from their substrate with EDTA, fixed with 3.7% paraformaldehyde, and incubated with a monoclonal mouse antibody directed against the external domain of the EGF receptor (528 IgG) at 37°C for 30 min. After washing, cells were incubated with goat anti-mouse immunoglobulin linked to phycoerythrin for a further 30 min at 37°C. Analysis was carried out on groups of 10,000 cells in a FACScanner (Becton Dickinson & Co., Mountain View, CA); data were analyzed with the accompanying LYSYS II software package. Histograms were taken through one round of smoothing. For indirect immunofluorescence, cells were fixed without dissociation and stained as described above; photographs were taken with an optiphot microscope (Nikon Inc., Garden City, NY) at a magnification of 400×. Parental NR6 cells lacking EGF receptors were analyzed in parallel to provide a control for background fluorescence.

SEM

For SEM analyses, cells were plated on half-inch glass coverslips and allowed to adhere. Where indicated, TPA (10 nM; Sigma Chemical Co., St. Louis, MO) was added either 24 h or 10 min before the experiment. The coverslips were transferred to warmed (37°C) media containing 10% dialyzed FBS and EGF (50 nM). Cells were maintained at 37°C until being fixed in 2.5% buffered glutaraldehyde (EMS, Fort Washington, PA) at room temperature for 1 h, postfixed in 2% OsO_4 for 1 h, and dehydrated in absolute ethanol. Peldri II (Ted Pella Inc., Irvine, CA), a sublimation dehydrant was used in place of critical point drying (Kennedy et al., 1989). After 1 h in a 1:1 mix of ethanol and Peldri II, the samples were transferred to liquid Peldri II, cooled to room temperature, and dried under vacuum for 12 h.

Table I. Comparison of the Sequence of Three Cloned Human EGF Receptor cDNAs

Base	Placental	Gen	UCSD	Effect
738	C	C	T	L160 – L
1806	C	C	C	No HindIII site
2547	G	A	A	Q763 – Q

The entire coding sequence from nucleotide 167 to 3880 was obtained using dideoxy chain terminating reactions. Reactions were run on parallel lanes of sequencing gels and the points of difference verified. Differences at bases 738 and 2547 do not change the predicted amino acids. None of the clones contain a HindIII restriction site at 1806 as indicated in the GenBank EGF receptor sequence.

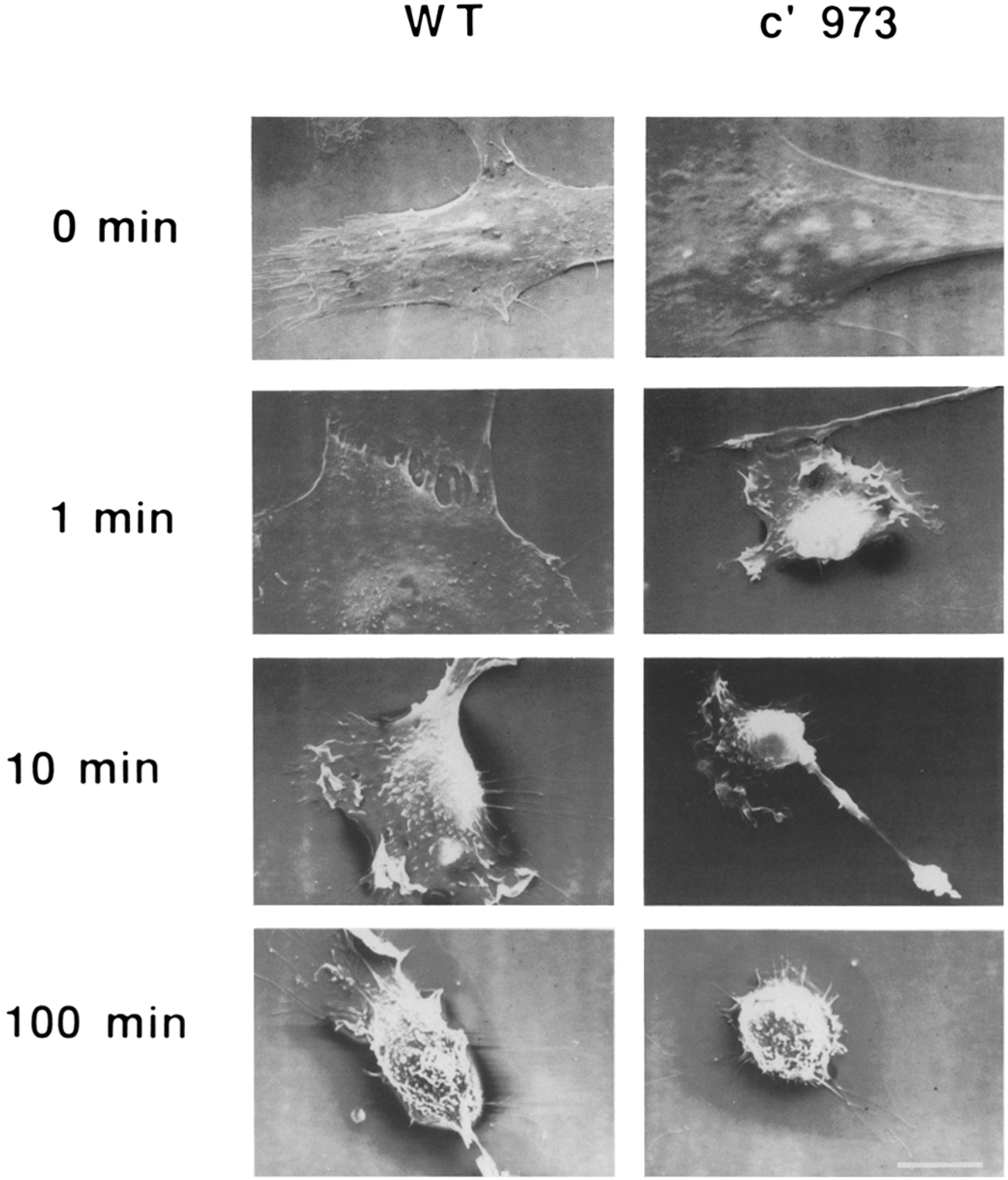

Figure 1. Scanning electron microscopic comparison of the rate and extent of EGF-induced morphological changes in NR6 cells expressing wild type and C′ terminal truncated EGF receptors. Cells expressing either the wild type (*WT*) or the c'973 T^{654} receptor (*c'973*) were exposed to 50 nM EGF for the indicated lengths of time. The cells were subsequently fixed, prepared, and visualized by SEM. Bar, 10 μm.

The coverslips were mounted on slotted specimen holders and coated with ~500 Å Au. A scanning electron microscope (model 360; Cambridge Instruments, Cambridge, U.K.) was used to image the cells. Representative individual cells were photographed at 2,500× magnification.

Quantitation of the Relative Cross-Sectional Area of Individual Cells

To quantitate the extent of morphological change of the cells under investi-

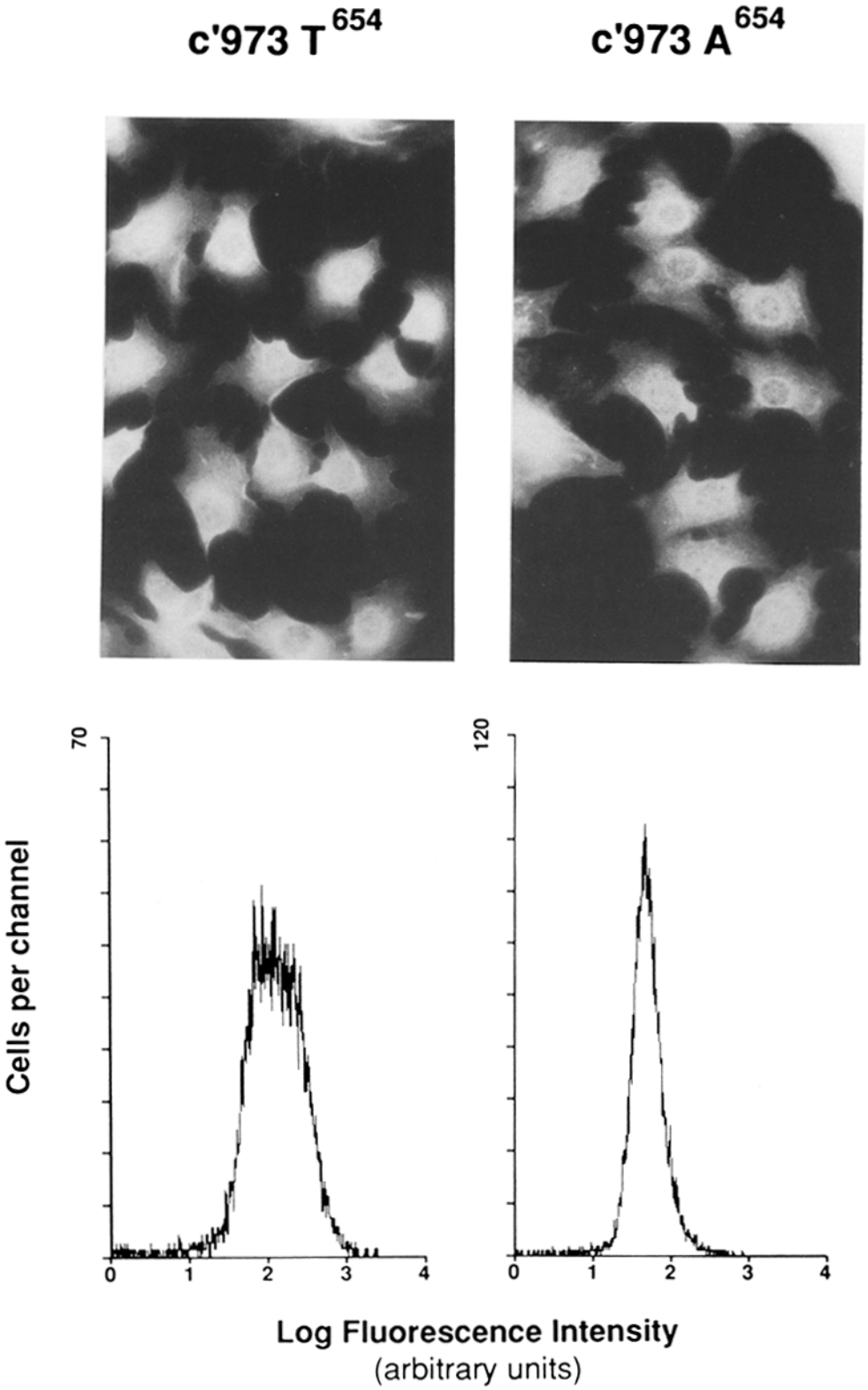

Figure 2. Expression of c'973 T^{654} and c'973 A^{654} EGF receptors in NR6 cells. Cells expressing either c'973 T^{654} or c'973 A^{654} EGF receptors were analyzed by indirect immunofluorescence and by fluorescence flow cytometry to ascertain expression of receptors. Cells were stained using the mouse mAb 528 IgG directed against the EGF receptor and visualized using phycoerythrin-conjugated goat anti-mouse Ig antibody.

gation, ten random individual cells were followed throughout the course of a 9-min treatment with EGF. Cells were plated onto glass coverslips 2 d before the experiment. TPA (10 nM) was added to the media of selected coverslips on the day before the experiment. They were then transferred to a microscope stage equilibrated to 37°C and photographed; EGF was added to a final concentration of 50 nM and photographs were taken of the same field of cells at 3, 6, and 9 min. Maximum cross-sectional area of the cells in the plane parallel to adherence was calculated. Experiments were performed pairwise, and were reproduced. Data from several experiments were pooled for calculation of changes in cross-sectional area and analysis using *t* test.

Results

C' Terminal Truncated EGF Receptors Elicit a More Rapid Change in Cell Morphology

EGF-induced morphological changes were compared be-

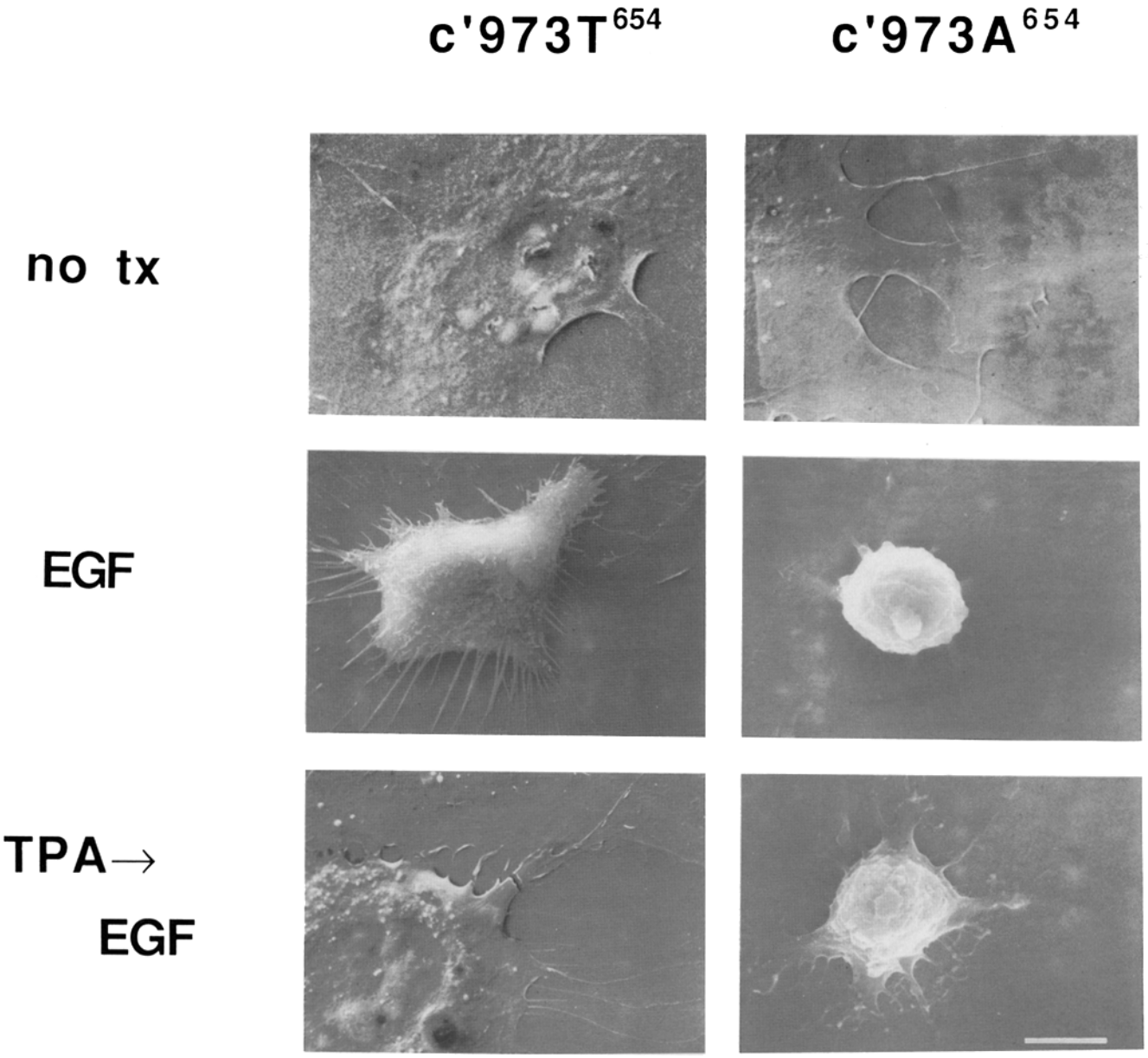

Figure 3. Effects of the phorbol ester TPA on EGF-induced morphologic changes in cells expressing T^{654} compared to A^{654} EGF receptors. NR6 cells expressed EGF receptors truncated after amino acid 973 and having either a threonine residue (*c'973 T^{654}*) or an alanine residue (*c'973 A^{654}*) at position 654. These cells were either mock treated (*no tx*), exposed to 50 nM EGF for 10 min (*EGF*), or to 10 nM TPA for 10 min followed by 50 nM EGF for 10 min (*TPA-EGF*). Bar, 10 μm.

tween cells expressing wild type and truncated c'973 T^{654} EGF receptors (Fig. 1). Cells expressing wild type EGF receptors began to show lamellipodial retraction by 10 min of exposure to 50 nM EGF, but a full response required more than 1 h. Cells expressing c'973 T^{654} EGF receptors behaved in a qualitatively similar manner, but retracted more rapidly. One minute after addition of EGF, lamellipodial retraction was evident, and there was an increase in the number of filopodia. These changes progressed at 5 min and by 10 min the c'973 T^{654} cells were rounded up with striking morphological alterations. The extent of morphological alteration at 100 min appeared similar for cells expressing the two EGF receptors. Because the time course of morphological changes in cells expressing wild type receptors overlaps the time course of ligand-induced receptor internalization and down regulation (Wiley and Cunningham, 1982; Glenney et al., 1988; Lund et al., 1990), cells expressing c'973 receptors were used in subsequent studies to investigate signal attenuation by mechanisms distinct from down regulation. The c'973 EGF receptor does not undergo signal attenuation by ligand-induced down regulation (Chen et al., 1989; Wells et al., 1990), making it more feasible to isolate other modes of signal attenuation. The more rapid and extensive morphological response of cells expressing c'973 EGF receptors presumably results from enhanced EGF-stimulated protein tyrosine kinase activity (Walton et al., 1990).

TPA Inhibits EGF-induced Morphological Changes in Cells Expressing T^{654} but Not A^{654} EGF Receptors

The threonine at amino acid 654 (T^{654}) was changed to ala-

nine (A^{654}) to remove a major site of receptor transmodulation by protein kinase C. Polyclonal lines of NR6 cells expressing either the c'973 T^{654} or c'973 A^{654} EGF receptor construction were established. Receptor expression in these lines was assessed to determine whether the reactions to ligand observed in a subset of these cells would be representative of the whole population. Immunofluorescence staining of both lines revealed that all cells expressed EGF receptor protein on the cell surface as an apparent homogenous population (Fig. 2). Parental NR6 cells served as a negative control (data not shown). Greater than 98% of the cells were recognized by the mAb to the EGF receptor when quantitated by flow cytometry. Comparison of the relative fluorescence of the two cell lines revealed a greater number of receptors and a somewhat broader distribution in T^{654} than A^{654} EGF receptors. Scatchard analysis confirmed this observation with $K_D = 1.9 \pm 0.2$ and 1.9 ± 0.3 nM and $B_{max} = 1.2 \times 10^5$ and 4.3×10^4 receptors $cell^{-1}$ in cells expressing c'973 T^{654} and c'973 A^{654} EGF receptors, respectively. Biphasic components of EGF binding are not included in these data because only a small number of high affinity EGF receptors were present. Comparison of either data set to the background fluorescence of the untransfected NR6 parental cell line indicated there was no detectable subpopulation lacking EGF receptors within the c'973 T^{654} or c'973 A^{654} receptor–expressing cell lines.

Fig. 3 shows that 10 min of EGF treatment induced significant morphological changes in NR6 cells expressing both c'973 T^{654} and c'973 A^{654} EGF receptors. After 10 min of exposure to EGF, the extent of morphological retraction was significantly greater in cells expressing A^{654} compared to T^{654} receptors. When cells were treated with 10 nM TPA for 10 min before treatment with EGF for 10 min, the EGF-induced morphological change was largely blocked in cells expressing T^{654} receptors. In contrast TPA failed to block the striking lamellipodial retraction, ruffling and rounding up of cells expressing A^{654} EGF receptors. Replacement of the single protein kinase C phosphorylation site at T^{654} with alanine thus completely abolished the inhibitory effect of TPA. The degree of TPA inhibition parallels the degree of inhibition by TPA of EGF-induced protein tyrosine kinase activity (Fig. 4). In cells expressing either c'973 T^{654} or c'973 A^{654} EGF receptors, EGF rapidly increased tyrosine phosphorylation of several cellular proteins. Treatment with 10 nM TPA for 10 min markedly decreased EGF-stimulated tyrosine phosphorylation of proteins in cells expressing c'973 T^{654} EGF receptors. In contrast TPA failed to affect EGF-stimulated protein tyrosine phosphorylation in cells expressing c'973 A^{654} EGF receptors.

Cells Expressing EGF Receptors Lacking a Protein Kinase C Phosphorylation Site at Residue 654 Respond More Rapidly and Extensively to EGF

If endogenously activated protein kinase C attenuates EGF receptor signaling, receptors that lack a feedback phosphorylation site would be expected to respond more rapidly and more extensively to EGF. The more extensive morphological change with EGF in cells expressing A^{654} compared to T^{654} receptors at 10 min (Fig. 3) suggested this was occurring. To examine this more closely, we compared the time-dependent extent of morphological changes in cells expressing T^{654} and A^{654} EGF receptors. Fig. 5 shows that, on

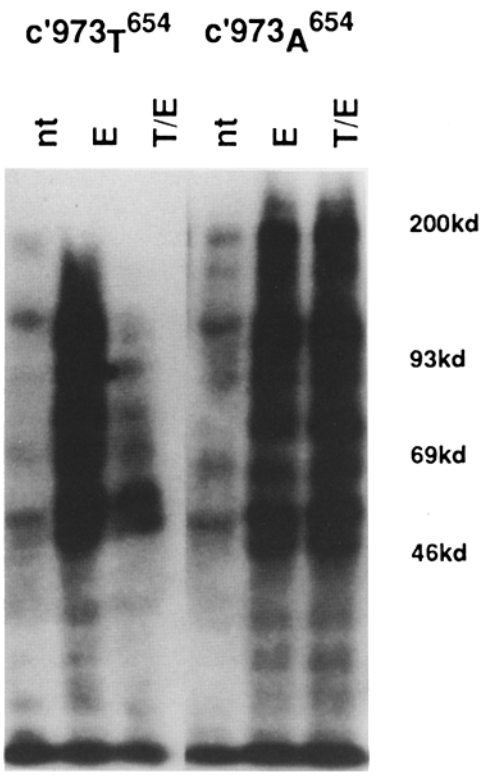

Figure 4. Effect of TPA on EGF-stimulated tyrosine kinase activity of T^{654} compared to A^{654} EGF receptors truncated at residue 973. NR6 cells ($\sim 5 \times 10^5$) expressing the indicated receptors were exposed to either media (*nt*), 10 nM EGF for 10 min (*E*), or 10 nM TPA for 10 min followed by EGF (*T/E*). The cells were then lysed in situ by the addition of hot Laemmli sample buffer and separated by 7.5% SDS-PAGE. The proteins were transferred to nitrocellulose. An ^{125}I-labeled mAb directed against phosphotyrosine was used to detect phosphotyrosine-containing proteins. c'973 T^{654}, receptor truncated after amino acid 973, with a threonine residue. c'973 A^{654}, receptor truncated after amino acid 973, with an alanine residue.

exposure to EGF, the cytoskeletons of both cells rearrange as demonstrated by lamellipodial retraction and increases in apparent height at various points along the cell margin. The change in morphology was more extensive in cells expressing A^{654} compared to T^{654} EGF receptors at each of the time points examined (3, 6, and 9 min after addition of 50 nM EGF). These results, which show that the rate and extent of EGF-induced morphological change is greater in cells expressing A^{654} receptors than in those expressing T^{654} receptors, suggest this mutation removes an inhibitory constraint.

To quantitate the morphological changes observed with SEM, individual cells were followed throughout the time course of EGF treatment by viewing and photographing randomly selected fields at low power (200×). When a cell retracts, the cross-sectional area parallel to the surface of attachment decreases. The cross-sectional area of individual cells was therefore measured at 3-min intervals after EGF addition. Fig. 6 shows that the rate and extent of decrease in cross-sectional area was significantly greater for cells expressing c'973 A^{654} than c'973 T^{654} EGF receptors. The changes observed with SEM are thus quantitative and representative of the entire population. The greater decrease in cross-sectional area in response to EGF in c'973 A^{654} EGF

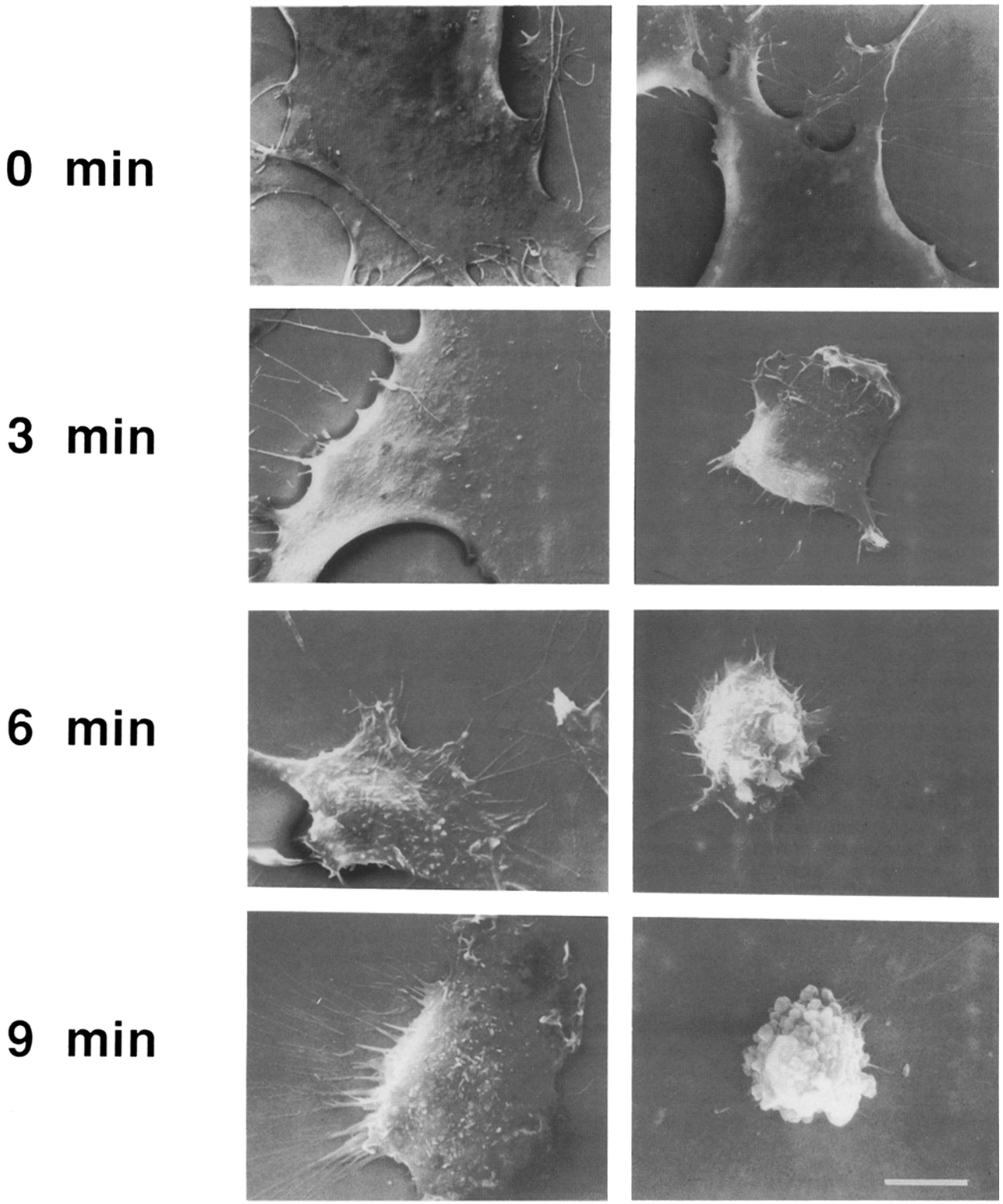

Figure 5. Time course of lamellipodial retraction in cells expressing T^{654} or A^{654} EGF receptors. Cells expressed EGF receptors truncated after amino acid 973 and had either a threonine residue (*c'973 T^{654}*) or an alanine residue (*c'973 A^{654}*) at position 654. They were exposed to 50 nM EGF for the indicated lengths of time and processed for SEM. Bar, 10 μm.

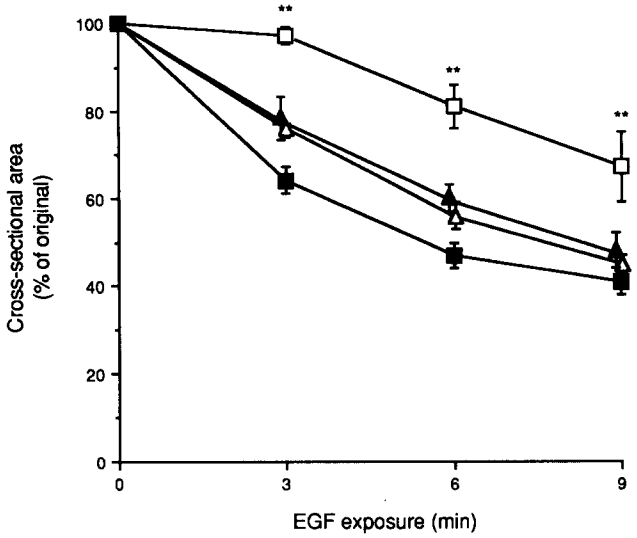

Figure 6. Cell retraction in response to EGF. NR6 cells, expressing the c'973 EGF receptor with either T or A at residue 654, were plated on coverslips. These were treated with 50 nM EGF. Random cells were followed individually at 3-min intervals by light microscopy at 200× magnification. The cross-sectional area of each cell was measured and scored as a percent of the area in the untreated state. □, NR6 cells expressing the T^{654} EGF receptor; ■, the same cells after 24-h exposure to 10 nM TPA; △, NR6 cells expressing the A^{654} EGF receptor; ▲, the same cells after 24-h exposure to 10 nM TPA. Results are mean ± standard error of means of 10 to 30 cells. **, $P < 0.001$ with respect to cells expressing c'973 A^{654} EGF receptors and to TPA-treated cell expressing either receptor.

receptor-expressing cells occurred despite a lower concentration of receptors compared to cells expressing c'973 T^{654} EGF receptors.

Down Regulation of Protein Kinase C Increases EGF-induced Morphological Changes

Analogous to removal of the T^{654} phosphorylation site in the EGF receptor, down regulation of protein kinase C by prolonged exposure of cells to TPA should relieve feedback inhibition via this enzyme. Exposure of NR6 cells to 10 nM TPA for 24 h reduced protein kinase C mass and activity more than 90% (data not shown) similar to that which occurs in parental 3T3 cells (Collins and Rozengurt, 1984; Ballester and Rosen, 1985). Cells expressing either c'973 T^{654} or A^{654} EGF receptors were exposed to 10 nM TPA for 24 h and then treated with EGF. The response of c'973 A^{654} cells was not affected by this treatment, confirming that protein kinase C did not affect the response of these cells to EGF (Fig. 6). In contrast, as shown in Fig. 7, 24-h treatment with TPA significantly enhanced EGF-stimulated morphological changes in NR6 cells expressing c'973 T^{654} EGF receptors. In TPA-pretreated cells morphological changes occurred earlier (3 and 6 min) and were more extensive. Quantitation of changes in cross-sectional area confirmed that down regulation of protein kinase C significantly enhanced morphological responses to EGF (Fig. 6).

Discussion

Activation of protein kinase C by the pharmacologic action of phorbol esters or by DAG produced in response to other hormones leads to decreased EGF receptor kinase activity (Cochet et al., 1984; Friedman et al., 1984; Downward et al., 1985; Lund et al., 1990). Transmodulation between signaling pathways also occurs through less well-defined mechanisms. Platelet-derived growth factor, which increases phosphorylation of the EGF receptor at T^{654}, also causes a decrease in high affinity EGF binding by a protein kinase C–independent mechanism (Olashaw et al., 1986; Davis and Czech, 1987). Interleukin 1 and tumor necrosis factor also act independently of protein kinase C to transmodulate the EGF receptor (Bird and Saklatvala, 1990). Palytoxin, a non-TPA type tumor promoter, may act through mediation of sodium fluxes to decrease both high and low affinity components of EGF binding (Wattenberg et al., 1989). A variety of mechanisms thus exists to coordinate responses among different signaling systems.

Similar mechanisms have been proposed to function as a negative feedback loop to attenuate EGF receptor signaling. King and Cooper (1986) demonstrated that some EGF receptor molecules were both self phosphorylated on tyrosine residues and at T^{654}. Because prior phosphorylation at T^{654} inhibits EGF receptor self phosphorylation (Cochet et al., 1984; Whiteley and Glaser, 1986; Decker et al., 1990; Lund et al., 1990), these findings were interpreted as evidence for a feedback loop that could attenuate further signaling by ligand-activated receptors. It has been difficult to critically test whether this proposed mechanism functions in vivo to attenuate EGF-induced responses because of the rapid and dominant effects of EGF-induced receptor internalization and down regulation. In the present studies we have used EGF receptor mutants defective in ligand-induced internalization and down regulation, and have examined the early morphologic alterations of lamellipodial retraction and membrane ruffling that reflect cytoskeletal responses to EGF. Several cytoskeletal proteins such as ezrin, spectrin (Bretscher, 1989), and calpactin II (Campos-Gonzales et al., 1990) are tyrosine phosphorylated in response to EGF and are thought to be involved in the morphologic changes induced by the growth factor.

Using c'973 T^{654} EGF receptors, TPA was shown to block EGF-induced morphologic changes and to inhibit EGF-stimulated protein tyrosine kinase activity in vivo. Replacement of the single major protein kinase C phosphorylation site with a non-phosphorylatable alanine residue rendered the mutant EGF receptor completely refractory to the inhibitory effects of TPA. This strongly supports previous findings that phosphorylation at T^{654} is the principal mechanism through which protein kinase C inhibits EGF receptors (Lin et al., 1986; Davis, 1988; Lund et al., 1990).

Although NR6 cells expressing c'973 T^{654} and c'973 A^{654} EGF receptors exhibit similar time response curves for the late response of EGF-stimulated cell growth (data not shown), the morphologic response to EGF was both more rapid and more extensive in cells expressing the A^{654} receptor. The enhanced EGF stimulated lamellipodial retraction and membrane ruffling in cells expressing c'973 A^{654} EGF receptors suggested that a negative feedback restraint, operative at T^{654}, was removed. This was further tested by down regulating protein kinase C in cells expressing c'973 T^{654} receptors. When protein kinase C was down regulated, the c'973 T^{654} receptor-expressing cells responded more rapidly

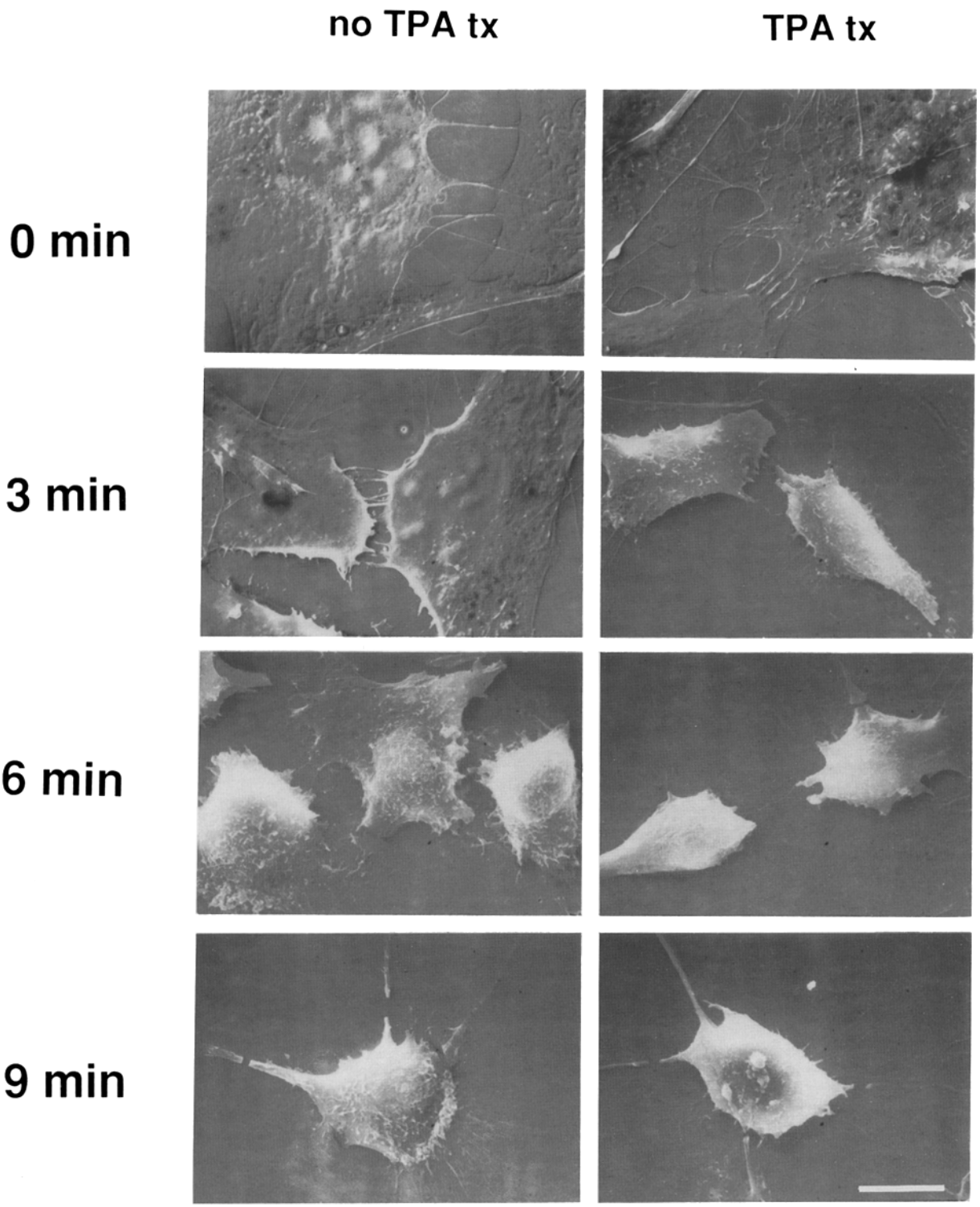

Figure 7. Effect of down regulation of protein kinase C on the response to EGF by the c'973 T^{654} EGF receptor-containing cells. NR6 cells expressing c'973 T^{654} EGF receptors were grown in the absence (*no TPA tx*) or presence (*TPA tx*) of 10 nM TPA for 24 h. Cells were subsequently exposed to 50 nM EGF for the indicated times. Bar, 10 μm.

and extensively, similar to those expressing c'973 A^{654} receptors. Thus, either elimination of the protein kinase C phosphorylation site (T^{654}) or down regulation of the enzyme led to a more rapid and extensive response to EGF. Together these results indicate that a feedback loop involving protein kinase C acts to attenuate EGF-stimulated responses.

The experimental system may underestimate the strength of the negative feedback loop regulating EGF receptor signaling. C' terminal truncation not only removes sequences required for ligand-induced down regulation but also removes sequences required for EGF-stimulated increases in cytosolic [Ca^{2+}] (Chen et al., 1989). The ability of ligand-activated EGF receptors to increase inositol phosphate production is also decreased by removal of C' terminal sequences (Q. C. Vega and G. N. Gill, unpublished observations). This suggests that EGF may activate protein kinase C by increasing DAG via mechanisms in addition to phospholipase C-γ activation. Wright et al. (1990) found that the rise in DAG in response to EGF stimulation is in large part because of hydrolysis of phosphatidylcholine, a process that can occur rapidly (Besterman et al., 1986). They implicate phospholipase D, which hydrolyzes phosphatidylcholine to choline and phosphatidic acid, the latter being converted to DAG. This receptor-mediated hydrolysis of phosphatidylcholine can lead to DAG production for the rapid activation of protein kinase C (Slivka et al., 1988). As reported for PDGF (Olashaw et al., 1986; Davis and Czech, 1987), other kinase pathways may also lead to phosphorylation at T^{654}. Use of c'973 A^{654} EGF receptors cannot distinguish such mechanisms, but the effects of down regulation of protein kinase C suggest that protein kinase C is strongly involved in EGF-induced receptor attenuation. If other kinases are involved, their principal target appears to be T^{654} (Countaway et al., 1990; Heisermann et al., 1990). These morphological data thus strongly support the hypothesis that protein kinase C is involved in physiologic regulation of EGF receptor signaling.

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