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Signal transduction by the epidermal growth factor receptor after functional desensitization of the receptor tyrosine protein kinase activity

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ABSTRACT **Previous work identified a protein kinase activity that phosphorylates the epidermal growth factor (EGF) receptor at Thr[669]. An assay for this protein kinase activity present in homogenates prepared from A431 human epidermoid carcinoma cells was developed using a synthetic peptide substrate corresponding to residues 663–681 of the EGF receptor (peptide T669). Here we report that a greater initial rate of T669 phosphorylation was observed in experiments using homogenates prepared from EGF- or phorbol ester-treated cells compared with control cells. EGF and 4β-phorbol 12-myristate 13-acetate (PMA) caused a 6-fold and a 2-fold increase in protein kinase activity, respectively. A kinetic analysis of T669 phosphorylation demonstrated that the increase in protein kinase activity observed was accounted for by an increase in V_{max}. To examine the interaction between protein kinase C and signal transduction by the EGF receptor, the effect of pretreatment of cells with PMA on the subsequent response to EGF was investigated. Treatment of cells with PMA caused >90% inhibition of the EGF-stimulated tyrosine phosphorylation of the EGF receptor and abolished the EGF-stimulated formation of soluble inositol phosphates. In contrast, PMA was not observed to inhibit the stimulation of T669 protein kinase activity caused by EGF. Thus, the apparent functional desensitization of the EGF receptor caused by PMA does not inhibit signal transduction mediated by the T669 protein kinase. Our results demonstrate that EGF receptor transmodulation alters the pattern of signal-transduction pathways that are utilized by the EGF receptor.**

Transmembrane signaling by the epidermal growth factor (EGF) receptor is acutely regulated by protein kinase C (reviewed in ref. 1). Activation of protein kinase C by treatment of cells with phorbol ester tumor promoters causes an inhibition of the high-affinity binding of EGF to cell surface receptors (2) and causes an inhibition of the receptor tyrosine protein kinase activity (3, 4). This process of acute regulation of EGF receptor function has been termed transmodulation (1). The molecular mechanism that accounts for transmodulation (5–10) and the role of transmodulation in signal transduction by the EGF receptor (11) are not understood. However, signaling by the transmodulated EGF receptor has been studied in detail. It has been demonstrated that EGF-stimulated phosphatidylinositol turnover and the generation of soluble inositol phosphates are inhibited by incubation of cells with phorbol ester (12–15). Phorbol ester also inhibits the increase in cytosolic Ca^{2+} caused by EGF without affecting basal cytosolic Ca^{2+} levels (12, 16). Furthermore, the EGF-stimulated Na^+/H^+ exchange across the plasma membrane is inhibited by phorbol ester (12, 17). Together, these data indicate that protein kinase C causes an acute desensitization of the function of the EGF receptor.

Recently we described a protein kinase activity in cell homogenates that phosphorylates the EGF receptor at threonine-669 (9). An assay for this protein kinase activity was developed using a synthetic peptide substrate corresponding to residues 663–681 of the EGF receptor (peptide T669). Treatment of cells with EGF caused a marked increase in the level of T669 protein kinase activity detected compared with control cells (9). The stimulation of T669 protein kinase activity caused by EGF indicates that this enzyme may have an important function in signal transduction. The purpose of the experiments described in this report was to examine the role of protein kinase C in the regulation of T669 protein kinase activity by EGF.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP, [γ-^{32}P]GTP, and ^{125}I-labeled goat anti-mouse immunoglobulin were from DuPont/NEN. [^{3}H]-Inositol was from Amersham. EGF was isolated from mouse submaxillary glands (18, 19). Membranes were prepared from A431 human epidermoid carcinoma cells (20). The monoclonal anti-phosphotyrosine antibody (PY20) was obtained from ICN. Phorbol ester was from Sigma. The synthetic peptides T669 (Glu-Leu-Val-Glu-Pro-Leu-Thr-Pro-Ser-Gly-Glu-Ala-Pro-Asn-Gln-Ala-Leu-Leu-Arg), KR($T^{669}S^{671}$) (Lys-Arg-Glu-Leu-Val-Glu-Pro-Leu-Thr-Pro-Ser-Gly-Glu-Ala-Pro-Asn-Gln-Ala-Leu-Leu-Arg), KR($T^{669}A^{671}$) (Lys-Arg-Glu-Leu-Val-Glu-Pro-Leu-Thr-Pro-Ala-Gly-Glu-Ala-Pro-Asn-Gln-Ala-Leu-Leu-Arg), and KR($A^{669}S^{671}$) (Lys-Arg-Glu-Leu-Val-Glu-Pro-Leu-Ala-Pro-Ser-Gly-Glu-Ala-Pro-Asn-Gln-Ala-Leu-Leu-Arg) were prepared using an Applied Biosystems model 430A machine (Peptide Synthesis Core Facility, University of Massachusetts Medical School). The synthetic peptide Lys-Arg-Thr-Leu-Arg-Arg was obtained from Peninsula Laboratories. A synthetic peptide based on the sequence of rabbit muscle glycogen synthase surrounding phosphorylation sites 3 (Pro-Arg-Pro-Ala-Ser$^{(3a)}$-Val-Pro-Pro-Ser$^{(3b)}$-Pro-Ser-Leu-Ser$^{(3c)}$-Arg-His-Ser-Ser-Pro) was the gift of T. Miller (University of Massachusetts Medical School). A431 human epidermoid carcinoma cells were obtained from G. Todaro (Oncogen, Seattle) and were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum (GIBCO).

Phosphorylation of Synthetic Peptides *in Vitro*. A431 cells were seeded in 35-mm wells and grown to a density of 10^6 cells per well. The cells were washed in serum-free medium (120 mM NaCl/6 mM KCl/1.2 mM $CaCl_2$/1 mM $MgCl_2$/30 μM bovine serum albumin/25 mM Hepes, pH 7.4) and incubated for 30 min at 37°C with 1 ml of the same medium. The cells were then treated without and with EGF or 4β-

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Abbreviations: EGF, epidermal growth factor; Ins-P_3, inositol trisphosphate; PMA, 4β-phorbol 12-myristate 13-acetate.

phorbol 12-myristate 13-acetate (PMA). The medium was aspirated and the cells were removed from the wells by scraping in 0.5 ml of 25 mM Hepes, pH 7.4/5 mM EGTA/50 mM NaF with 10 μg of leupeptin per ml at 0°C and were homogenized by 10 passages through a 26-gauge needle. Phosphorylation reactions were performed at 22°C using 5 μl of the cell extract, 25 μl of 50 mM Hepes, pH 7.4/20 mM $MgCl_2$, and 10 μl of synthetic peptide. The phosphorylation reaction was initiated by the addition of 10 μl of 50 μM [γ-^{32}P]ATP (100 μCi/nmol, 1 μCi = 37 kBq) (unless otherwise indicated). In experiments designed to investigate GTP as a substrate, [γ-^{32}P]ATP was replaced with [γ-^{32}P]GTP (100 μCi/nmol). The phosphorylation reaction was terminated by the addition of 10 μl of 90% formic acid and the phosphorylated synthetic peptides were isolated by thin-layer electrophoresis (9). The phosphorylated peptides were identified by autoradiography and the incorporation of radioactivity into the peptides was quantitated by liquid scintillation counting.

Autophosphorylation of the EGF Receptor in Intact Cells. A431 cells were grown in 35-mm dishes to a density of 10^6 cells per well. The cells were then washed with serum-free medium and subsequently incubated at 37°C for 30 min. The EGF receptors were isolated by immunoprecipitation and polyacrylamide gel electrophoresis (21). The state of tyrosine phosphorylation of the EGF receptors was investigated by a Western blot procedure (22) using a monoclonal anti-phosphotyrosine antibody (PY20) and an ^{125}I-labeled goat anti-mouse immunoglobulin second antibody.

Quantitation of Inositol Trisphosphate (Ins-P_3). A431 cells were seeded in 35-mm wells and grown to 10^6 cells per well. [^{3}H]Inositol (25 μCi/ml) was added to the culture medium during the last 24 hr of incubation. The cells were then washed with serum-free medium and subsequently incubated at 37°C for 30 min in 1 ml of serum-free medium. The cells were treated for defined times with EGF and PMA. Subsequently, the medium was aspirated and 1 ml of 10% (wt/vol) trichloroacetic acid (4°C) was added to each well. The soluble acid supernatants of the cells were extracted with ether and analyzed by Dowex-1 chromatography (23). The Ins-P_3 fraction (containing inositol trisphosphates and tetrakisphosphates) eluted from the column with 1 M ammonium formate/0.1 M formic acid was collected and the radioactivity associated with this fraction was measured by liquid scintillation counting.

RESULTS

Regulation of T669 Protein Kinase Activity by EGF and Phorbol Ester. The time course of phosphorylation of the synthetic peptide T669 in assays using homogenates prepared from A431 cells showed that phosphorylation of the peptide increased linearly with time for 15 min (Fig. 1). The rate of peptide phosphorylation was increased if the cells were treated with EGF or PMA prior to homogenization. Incubation of cells with 100 nM EGF for 15 min caused an ≈6-fold increase in the initial rate of peptide phosphorylation compared with the rate for homogenates prepared from control cells. Treatment with 100 nM PMA for 15 min caused an ≈2-fold increase in the initial rate of peptide phosphorylation compared with control cells (Fig. 1). Half-maximal effects were observed after treatment of the cells with 1 nM PMA (Fig. 2*A*). Stimulation of T669 protein kinase activity occurred within 5 min of treatment with 100 nM PMA, and the activity remained elevated for 60 min (Fig. 2*B*).

The primary amino acid sequence surrounding Thr^{669} contains three proline residues. The proximity of these proline residues to Thr^{669} is unusual for a site of protein phosphorylation (24). However, glycogen synthase kinase 3 has been reported to phosphorylate glycogen synthase at sites located within a proline-rich primary sequence (25). To assess the

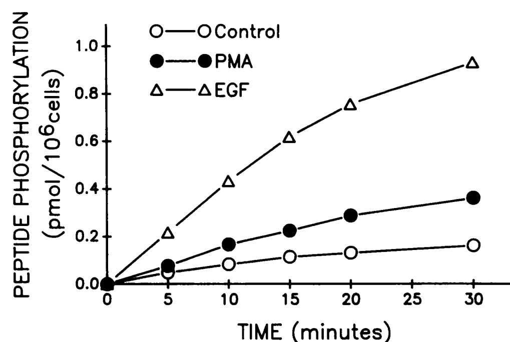

FIG. 1. Time course of phosphorylation of peptide T669. A431 cells were incubated without or with 100 nM PMA or 100 nM EGF for 15 min. Homogenates were prepared from these cells and incubated with the synthetic peptide T669 (0.5 mg/ml) and [γ-^{32}P]-ATP (10 μM). Similar results were obtained in three separate experiments.

specificity of the T669 protein kinase activity detected in homogenates prepared from PMA- and EGF-treated A431 cells, experiments were performed using a proline-rich synthetic peptide based on the sequence surrounding phosphorylation sites 3 of glycogen synthase (see *Experimental Procedures*). Although this peptide was phosphorylated by a protein kinase activity present in cell homogenates, no significant effect of PMA or EGF treatment of A431 cells was observed on the apparent K_m or V_{max} of phosphorylation of the glycogen synthase peptide (Table 1).

The synthetic peptide T669 contains two potential phosphorylation sites, a threonine and a serine residue. These residues correspond to Thr^{669} and Ser^{671} of the EGF receptor. [^{32}P]Phospho amino acid analysis of peptide T669 phosphorylated *in vitro* indicates the presence of [^{32}P]phosphothreonine and a trace amount of [^{32}P]phosphoserine (9). To assess the

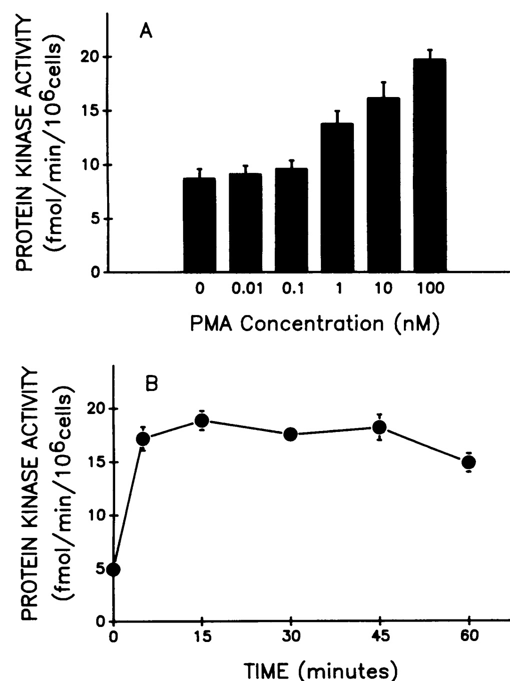

FIG. 2. Regulation of T669 protein kinase activity by PMA. A431 cells were incubated with various concentrations of PMA for 30 min (*A*) or with 100 nM PMA for various times (*B*). The protein kinase activity in homogenates prepared from these cells was assayed using peptide T669 (0.5 mg/ml) and [γ-^{32}P]ATP (10 μM) as substrates. Assays were terminated after 10 min of incubation at 22°C. Results are means ± SD of three separate experiments.

Table 1. Apparent kinetic constants (K_m, mM; V_{max}, fmol/min per 10^6 cells) for the phosphorylation of synthetic peptides

Peptide	Control		PMA		EGF	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Glycogen synthase sites 3	2.1 ± 0.8	36 ± 13	3.0 ± 0.7	47 ± 2.3	2.5 ± 0.7	46 ± 13
T669	1.0 ± 0.4	19 ± 6.0	0.7 ± 0.2	36 ± 7.0	1.0 ± 0.2	118 ± 16
KR($T^{669}S^{671}$)	0.28 ± 0.04	8.2 ± 1.6	0.35 ± 0.02	15 ± 4.0	0.5 ± 0.1	51 ± 11
KR($T^{669}A^{671}$)	0.8 ± 0.3	5.2 ± 2.3	0.8 ± 0.3	8.3 ± 2.3	0.73 ± 0.22	23 ± 1.4
KR($A^{669}S^{671}$)	ND	ND	ND	ND	ND	ND

A431 cells were incubated without or with 100 nM EGF or 100 nM PMA for 15 min. The protein kinase activity in homogenates prepared from these cells was assayed using various concentrations of synthetic peptides and 10 μM [γ-^{32}P]ATP as substrates. The peptides used were based on the primary sequence surrounding glycogen synthase phosphorylation sites 3 and EGF receptor Thr^{669} (peptide T669). KR($T^{669}S^{671}$) is T669 with an N-terminal Lys-Arg extension. Peptides containing point mutations at Ser^{671} or Thr^{669} are designated KR($T^{669}A^{671}$) and KR($A^{669}S^{671}$). The assays were terminated after 10 min at 22°C. Results are means ± SD of triplicate determinations obtained by curve-fitting to the Michaelis–Menten formalism. This was achieved by weighted nonlinear regression employing "robust" methods for the modification of residuals (26). No phosphorylation of the KR($A^{669}S^{671}$) peptide was observed, so the kinetic rate constants were not determined (ND).

role of Thr^{669} and Ser^{671} a set of synthetic peptides was constructed in which these residues were replaced with alanine. No phosphorylation of the Ala^{669}, Ser^{671} peptide was detected, but both the Thr^{669}, Ala^{671} and the Thr^{669}, Ser^{671} peptides were phosphorylated (Table 1). Comparison of the kinetic constants for the phosphorylation of the Thr^{669}, Ala^{671} and Thr^{669}, Ser^{671} peptides indicates that the substitution of alanine for Ser^{671} caused a decrease in the apparent V_{max} and an increase in the apparent K_m for peptide phosphorylation (Table 1).

Mechanism of Regulation of T669 Protein Kinase Activity. The stimulation of T669 protein kinase activity in cells treated with PMA or EGF could be caused by either a change in K_m of the enzyme for substrates or an increase in V_{max}. To distinguish between these possibilities the apparent K_m and V_{max} values were measured for homogenates prepared from control, EGF-treated, and PMA-treated cells. No significant change in K_m for the synthetic peptide T669 was observed (Table 1). However, PMA and EGF were observed to cause a 1.8-fold and a 6.2-fold increase in the measured V_{max}, respectively (Table 1). A similar result was obtained in experiments designed to measure the K_m for ATP (Table 2). GTP was found to be a poor substrate for the T669 protein kinase activity (Table 2). These data indicate that the increase in T669 protein kinase activity caused by PMA or EGF can be accounted for by an increase in the enzyme V_{max}.

Role of Protein Kinase C During Stimulation of T669 Protein Kinase Activity by EGF. As PMA was observed to cause an increase in T669 protein kinase activity, it was possible that protein kinase C was mediating the stimulation caused by EGF. To test this hypothesis, the effect of down-regulation of protein kinase C to alter the ability of EGF to increase T669 protein kinase activity was examined. Protein kinase C was down-regulated in A431 cells by incubation of cells with 1 μM PMA for 48 hr. No protein kinase C activity was detected in homogenates prepared from these cells using the synthetic peptide Lys-Arg-Thr-Leu-Arg-Arg as a substrate (21). Incubation of control cells with PMA or EGF caused a 1.8 ± 0.04-fold and a 7.6 ± 0.7-fold (mean ± SD, $n = 3$) stimulation of T669 protein kinase activity, respectively. No significant stimulation of T669 protein kinase activity was observed after PMA treatment of down-regulated cells. In contrast, EGF increased T669 protein kinase activity 5.1 ± 0.4-fold (mean ± SD, $n = 3$) in down-regulated cells. Thus, down-regulation of protein kinase C abolished the ability of PMA to increase T669 protein kinase activity. However, EGF increased T669 protein kinase activity in cells that lacked detectable protein kinase C. These data indicate that EGF can stimulate T669 protein kinase activity in A431 cells by a protein kinase C-independent mechanism.

Effect of PMA on Signal Transduction by the EGF Receptor. The stimulation of T669 protein kinase activity caused by EGF was compared with the effects of EGF to increase tyrosine phosphorylation and phosphatidylinositol turnover. Incubation of A431 cells with PMA for 20 min caused an inhibition of EGF-stimulated tyrosine phosphorylation (Fig. 3*A*) and EGF-stimulated phosphatidylinositol turnover (Fig. 3*B*). However, PMA treatment did not prevent the stimulation of T669 protein kinase activity by EGF action (Fig. 3*C*).

In further experiments, the time course of regulation of T669 protein kinase activity caused by EGF following treatment of A431 cells with PMA was examined. A431 cells were incubated without or with 100 nM PMA for 20 min prior to incubation for various times with 100 nM EGF. A marked increase in T669 protein kinase activity was observed within 5 min of addition of EGF to control and phorbol ester-treated cells (Fig. 4). The increased T669 protein kinase activity was maintained for 30 min after EGF treatment (Fig. 4). Investigation of the EGF dose–response indicated that EGF caused an increase in T669 protein kinase activity to a similar extent when control and phorbol ester-treated cells were compared (data not shown). Consistent with the effect of PMA to cause a decrease in the apparent affinity of the EGF receptor (2), the concentration of EGF required to cause half-maximal stimulation of T669 protein kinase activity was increased by PMA treatment of A431 cells (data not shown).

DISCUSSION

A protein kinase activity that phosphorylates the EGF receptor at Thr^{669} has been described (9). An assay for this protein kinase activity was developed using a synthetic

Table 2. Comparison of the apparent kinetic constants (K_m, μM; V_{max}, fmol/min per 10^6 cells) for peptide T669 phosphorylation using ATP and GTP as substrates

Nucleotide	Control		PMA		EGF	
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
ATP	40 ± 3.3	14 ± 3	54 ± 12	25 ± 2	59 ± 10	112 ± 40
GTP	>200	0.58 ± 0.08	>200	0.88 ± 0.2	>200	1.0 ± 0.4

A431 cells were incubated without or with 100 nM EGF or 100 nM PMA for 15 min. The protein kinase activity in homogenates prepared from these cells was assayed using various concentrations of [γ-^{32}P]ATP or [γ-^{32}P]GTP and synthetic peptide T669 (0.5 mg/ml) as substrates. Assays were terminated after 10 min at 22°C. Results are means ± SD of triplicate determinations obtained by curve-fitting to the Michaelis–Menten formalism, achieved as described for Table 1.

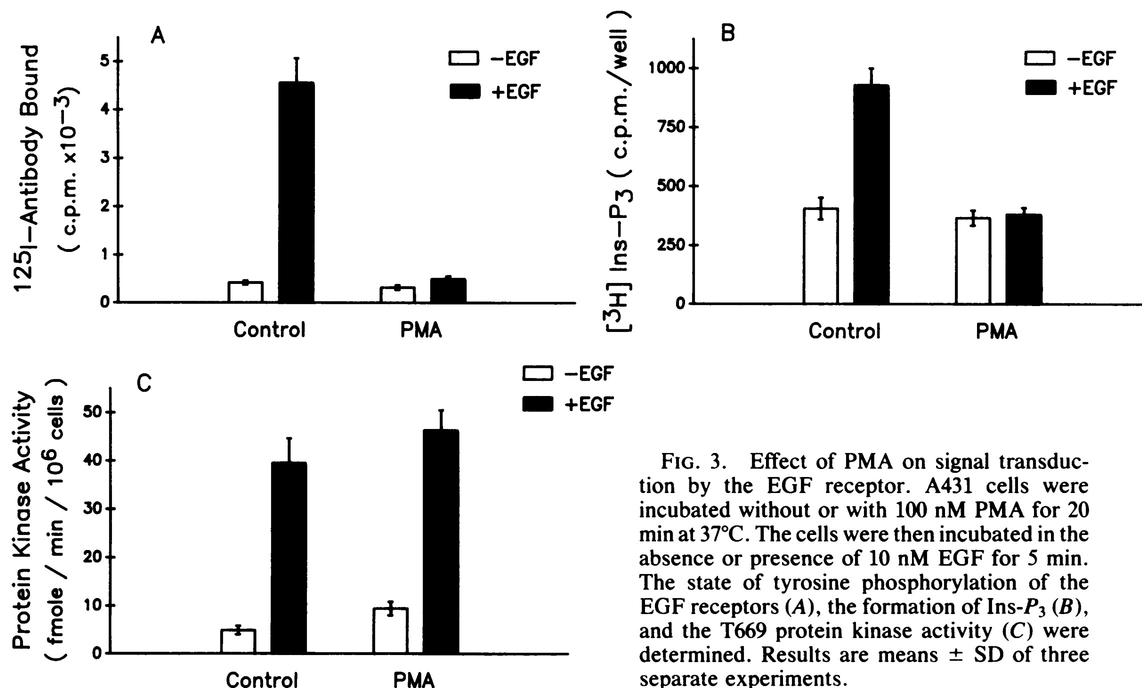

FIG. 3. Effect of PMA on signal transduction by the EGF receptor. A431 cells were incubated without or with 100 nM PMA for 20 min at 37°C. The cells were then incubated in the absence or presence of 10 nM EGF for 5 min. The state of tyrosine phosphorylation of the EGF receptors (*A*), the formation of Ins-P_3 (*B*), and the T669 protein kinase activity (*C*) were determined. Results are means ± SD of three separate experiments.

peptide substrate corresponding to residues 663–681 of the EGF receptor (peptide T669). In the present study, we have further characterized the T669 protein kinase activity in A431 human epidermoid carcinoma cells. PMA caused an ≈2-fold stimulation of T669 protein kinase activity (Fig. 2). To determine the mechanism of this increased protein kinase activity, a kinetic analysis was performed on homogenates of control cells and cells treated with PMA or EGF. The measured K_m for the synthetic peptide T669 (0.74–0.98 mM, Table 1) and ATP (40–60 μM, Table 2) did not change significantly under any of the conditions tested. The apparent V_{max} increased 2-fold in cells treated with PMA and 6-fold in cells treated with EGF (Tables 1 and 2). The increase in T669 protein kinase activity observed after treatment of cells with EGF or with PMA can therefore be accounted for by an increase in V_{max}. These data are consistent with the hypotheses that EGF and PMA cause either increased activity of the T669 protein kinase or recruitment of inactive enzymes to an active population of T669 protein kinase. Further experimental work is required to distinguish between these hypotheses.

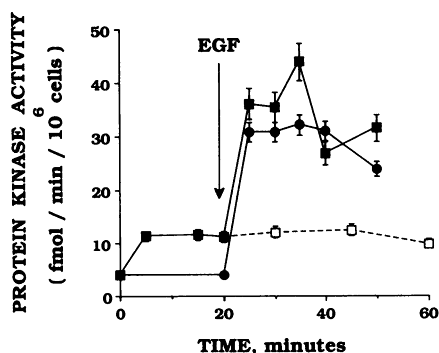

FIG. 4. Effect of PMA on the time course of regulation of T669 protein kinase activity by EGF. A431 cells were incubated without (●) or with (□, ■) 100 nM PMA for 20 min. The cells were then incubated without (□) or with (●, ■) 100 nM EGF for various times. The protein kinase activity in homogenates prepared from these cells was assayed using peptide T669 (0.5 mg/ml) and [γ-^{32}P]ATP (10 μM) as substrates. Assays were terminated after 10 min at 22°C. Results are means ± SD of three separate experiments.

Role of Protein Kinase C. Treatment of A431 cells with PMA caused an ≈2-fold increase in the measured T669 protein kinase activity compared with control cells (Fig. 2). Down-regulation of protein kinase C abolished the effect of PMA to stimulate T669 protein kinase activity. In contrast, the effect of EGF to increase T669 protein kinase activity was observed in experiments using both control cells and protein kinase C-down-regulated cells. These data indicate that, while a protein kinase C-dependent pathway of regulation is present in A431 cells, EGF is able to regulate T669 protein kinase activity by a pathway that is independent of protein kinase C. As EGF stimulates protein kinase C in A431 cells (27), it is likely that EGF regulates T669 protein kinase activity by both a protein kinase C-dependent and a protein kinase C-independent pathway.

Signal Transduction by the EGF Receptor After Protein Kinase C Activation. Incubation of cultured fibroblasts and A431 cells with tumor-promoting phorbol ester causes EGF receptor transmodulation (1). It has been reported that the high-affinity binding of EGF to the receptor is markedly reduced (2) and that the EGF-stimulated tyrosine protein kinase activity of the receptor is inhibited (3, 4, 6). Associated with this acute desensitization of EGF receptor function is an inhibition of signal transduction by the EGF receptor. EGF-stimulated phosphatidylinositol turnover (12–15), Na^+/H^+ exchange (12, 17), and Ca^{2+} fluxes (12, 16) are blocked by treatment of cells with phorbol ester. Furthermore, the EGF-stimulated proliferation of human fibroblasts (28) and murine NIH 3T3 cells (7) is inhibited by treatment of cells with phorbol ester.

As the treatment of cultured cells with phorbol ester has been reported to cause the desensitization of EGF receptor function (1–8, 12–17), we examined the effect of phorbol ester treatment on the regulation of T669 protein kinase activity by EGF. Treatment of A431 cells for 20 min with PMA caused an increase in the concentration of EGF required for the half-maximal stimulation of the T669 protein kinase activity (data not shown). This change in the EGF dose–response is consistent with the effect of PMA to decrease the apparent affinity of the EGF receptor (2). However, PMA was not found to inhibit the maximal extent of stimulation of T669 protein kinase activity caused by EGF (Figs. 3 and 4). This result indicates that PMA does not cause desensitization of

EGF receptor signaling mediated by the T669 protein kinase. This contrasts with previous observations that phorbol ester causes the desensitization of EGF receptor function at all concentrations of EGF tested (1–8, 12–17).

An hypothesis that can account for the apparent desensitization of the EGF receptor caused by PMA is that the phosphorylation of the receptor at Thr^{654} by protein kinase C causes an inhibition of the EGF receptor tyrosine protein kinase activity (3, 4, 6, 10). Mutant EGF receptors that lack tyrosine protein kinase activity are defective in signal transduction (29, 30). The inhibition (>90%) of the EGF receptor tyrosine protein kinase activity caused by PMA can therefore account for the effect of PMA to inhibit EGF-stimulated phosphatidylinositol turnover (12–15), Na^+/H^+ exchange (12, 17), and Ca^{2+} fluxes (12, 16). However, this inhibition of the EGF receptor tyrosine protein kinase activity is not sufficient to inhibit signal transduction mediated by the T669 protein kinase (Fig. 3). These data suggest that the threshold of tyrosine phosphorylation required for EGF stimulation of the T669 protein kinase is lower than that required for the activation of other signaling pathways.

Conclusions. Transmodulation of the EGF receptor has been reported to cause the desensitization of EGF receptor function (1–8, 12–17). The observation reported here that the transmodulated EGF receptor is capable of signal transduction (mediated by the T669 protein kinase) indicates that transmodulation does not result in the complete desensitization of EGF receptor function. Instead, the results of this study demonstrate that EGF receptor transmodulation changes the pattern of signal-transduction pathways that are utilized by the EGF receptor. This conclusion may be physiologically significant because EGF receptor transmodulation caused by platelet-derived growth factor results in the synergistic stimulation of the growth of cells cultured with EGF.

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