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Pertussis toxin or phorbol 12-myristate 13-acetate can distinguish between epidermal growth factor- and angiotensin-stimulated signals in hepatocytes

(mitogens/inositol lipids/GTP-binding proteins/calcium ion/protein phosphorylation)

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ABSTRACT **Epidermal growth factor (EGF) causes rapid increases in free intracellular Ca^{2+} and stimulates the phosphorylation of 11 cytosolic proteins in hepatocytes. Ten of the 11 cytosolic proteins altered by EGF are identical to those affected by angiotensin II, a hormone that stimulates the breakdown of phosphatidylinositol 4,5-bisphosphate. An increase in the phosphorylation of the other protein, spot c (M_r = 36,000, pI = 5.5), is observed only with EGF. Treatment of intact rats with pertussis toxin to ADP-ribosylate N_i, the inhibitory GTP-binding protein of the adenylate cyclase complex, abolished the effect of EGF on Ca^{2+} mobilization and on the phosphorylation of the 10 proteins affected in common with angiotensin II. This treatment had minimal effects on the ability of EGF to stimulate the phosphorylation of its unique substrate, spot c. In marked contrast, modification of N_i did not block the ability of angiotensin II to stimulate Ca^{2+} mobilization or protein phosphorylation. Pretreatment of normal hepatocytes with 4β-phorbol 12-myristate 13-acetate blocked all responses to EGF, including the increased phosphorylation of spot c, but had no effect on the responses to angiotensin II. These results imply that N_i or a similar pertussis toxin substrate may mediate the apparent effects of EGF on phosphatidylinositol breakdown and that protein kinase C may regulate a site in the transduction pathway. Angiotensin II appears to use a different signal transduction mechanism to stimulate phosphatidylinositol metabolism in hepatocytes.**

The exact sequence of events leading to the biological effects of epidermal growth factor (EGF) remains obscure. Most recent studies have concentrated on the tyrosine kinase activity of the EGF receptor, either at the level of the membrane or within the cytoplasm (1). However, EGF and other growth factors also stimulate phosphatidylinositol breakdown (2–4), generating inositol trisphosphate ($InsP_3$) and diacylglycerol ($acyl_2Gro$) as intracellular messengers (4). $InsP_3$ releases Ca^{2+} from intracellular stores, activating Ca^{2+}/calmodulin-sensitive protein kinases, and $acyl_2Gro$ activates protein kinase C (4–6). Since these enzymes may also control cell growth, increasing attention is being focused on inositol lipid metabolism as a possible regulator of cellular proliferation (4, 6, 7). The two signaling systems may also interact, in that phorbol esters, putatively by activating protein kinase C, can modify the responses of a number of cells to EGF (1, 8, 9).

One well-characterized model of transmembrane signaling is provided by the adenylate cyclase complex. In this system, two guanine-nucleotide-binding proteins, N_s and N_i, mediate the effects of stimulatory and inhibitory receptors on the catalytic moiety that generates cyclic AMP (10). Recent evidence suggests that similar GTP-binding proteins may mediate the effects of agonists in other signal-transduction systems (11). In fact, experiments using pertussis toxin as a probe suggest that N_i may mediate the effects of certain agonists that stimulate the phosphatidylinositol response (12–16).

Both EGF and angiotensin II stimulate phosphatidylinositol breakdown in certain cells (2, 4, 17). The present study compared the ability of these hormones to generate the $InsP_3$ (Ca^{2+}) and $acyl_2Gro$ signals in hepatocytes by using quin2 and changes in the phosphorylation state of certain proteins to monitor the response. While both hormones were found to generate the Ca^{2+} and $acyl_2Gro$ signals, either pertussis toxin or 4β-phorbol 12-myristate 13-acetate (PMA) was able to block the responses to EGF without affecting the response to angiotensin II. These results suggest that there are differences in the mechanism by which EGF and angiotensin II generate $InsP_3$ and $acyl_2Gro$ in hepatocytes.

EXPERIMENTAL PROCEDURES

General Methods. Isolated liver cells were prepared from normal or 72-hr pertussis-toxin-treated, 200- to 250-g, fasted male Wistar rats (18). Rats were treated with pertussis toxin by intraperitoneal injection of 50 μg of toxin per 100 g of body weight (19). An aliquot of each preparation of normal or intoxicated cells was loaded with quin2 to monitor the effects of hormones on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), loaded with $[^{32}P]PO_4^{3-}$ to monitor the effects of hormones on protein phosphorylation, or frozen prior to isolating plasma membranes.

$[Ca^{2+}]_i$ was measured with quin2 as described by Charest *et al.* (20) with the following modifications: the amount of quin2 tetrakis(acetoxymethyl) ester (quin2/AM) loaded per cell was reduced; the gelatin in the medium was replaced with crystalline bovine serum albumin at 2 mg/ml; and digitonin at 30 μg/ml was used to permeabilize the cells for estimation of $[Ca^{2+}]_i$ as described by Tsien *et al.* (21). Fluorescence readings were obtained at 1-sec intervals by using a SPEX Fluorolog model 111C spectrofluorometer, stored, and plotted with the computer integral to the instrument. The data in Fig. 1 are copies of the traces as plotted by the computer. The cells in the cuvette were maintained at 37°C, stirred gently and gassed with a steady supply of 95% O_2/5% CO_2.

The effects of hormones on the phosphorylation state of cytosolic proteins in ^{32}P-labeled intact hepatocytes was measured by resolving cytoplasmic extracts on two-dimen-

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Abbreviations: EGF, epidermal growth factor; PMA, 4β-phorbol 12-myristate 13-acetate; $acyl_2Gro$, diacylglycerol; $InsP_3$, L-*myo*-inositol 1,4,5-trisphosphate; quin2, 2-{[2-bis(carboxymethyl)amino-5-methylphenoxy]methyl}-6-methoxy-8-bis(carboxymethyl)aminoquinoline; $[Ca^{2+}]_i$, free intracellular Ca^{2+} concentration.

sional gels, visualizing the ^{32}P-labeled proteins with autoradiography, and quantitating the optical density information on the films (22, 23). The activity of glycogen phosphorylase was measured as described (23).

Membranes were prepared from isolated hepatocytes by flotation on sucrose gradients as described (19). To determine the degree of prior modification of N_i catalyzed by pertussis toxin *in vivo*, the N_i in the membrane was extracted with sodium cholate and ADP-ribosylated in solution with [^{32}P]NAD^+ and activated pertussis toxin as described by Pobiner *et al.* (19).

Suppliers. Quin2/AM was purchased from Calbiochem. EGF was purchased from Sigma or Collaborative Research (Lexington, MA). The sources of all other reagents have been published (23).

Expression of Results. Data presented in Figs. 1–3 are representative of four or five experiments. Averaged data are presented as the means ± SEM. Statistical evaluation was performed by the paired *t* test.

RESULTS

Effects of Pertussis Toxin and PMA on EGF- and Angiotensin II-Stimulated Ca^{2+} Signals. In hepatocytes, hormone-induced changes in $[Ca^{2+}]_i$ closely follow the breakdown of phosphatidylinositol 4,5-bisphosphate and the generation of $InsP_3$ (24). Therefore, $[Ca^{2+}]_i$ was monitored with quin2 to provide a continuous record of the phosphatidylinositol response. As expected, both EGF and angiotensin II caused a marked increase in $[Ca^{2+}]_i$ in normal hepatocytes (Fig. 1 *Top*). The rapid rise in $[Ca^{2+}]_i$ was not attenuated by adding up to a 5- to 10-fold excess of EGTA (over extracellular Ca^{2+}) to the incubation medium, suggesting that the initial Ca^{2+} signal comes from internal stores (data not shown). In keeping with its effects on $[Ca^{2+}]_i$, EGF also increased phosphorylase activity about 3.3 ± 0.2-fold over a basal level of 12 nmol of glucose-1-PO_4 per mg of protein per min (n = 3). About 5 nM EGF caused half-maximal activation of phosphorylase, with maximal effects occurring at about 50 nM. Since the intent of the study was to examine possible inhibitors of the EGF response, maximal doses of EGF (400 ng/ml or 66 nM) were used in all subsequent experiments. While the effects of EGF and angiotensin II on $[Ca^{2+}]_i$ are qualitatively similar, the data in Table 1 show that maximal doses of angiotensin II generate a faster and larger increase in the Ca^{2+} signal.

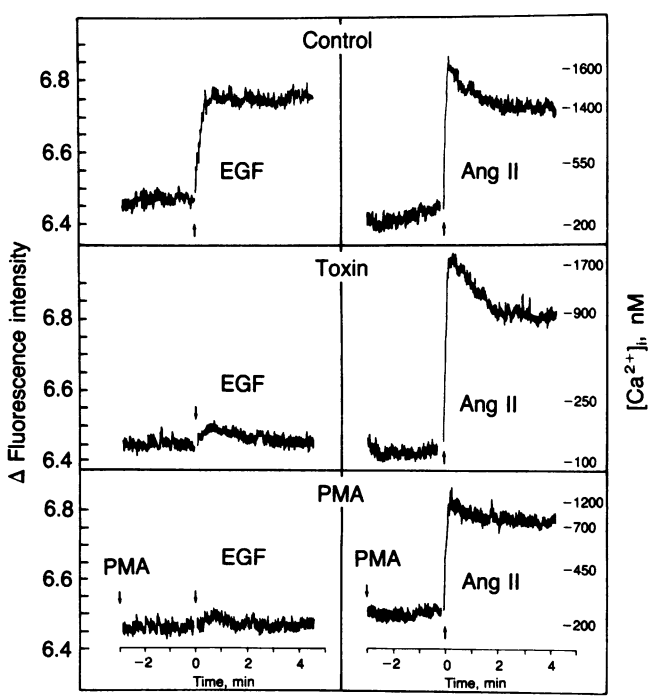

FIG. 1. Response of quin2-loaded hepatocytes to EGF and angiotensin II. Traces represent typical quin2 fluorescence changes after addition of 66 nM EGF (400 ng/ml) or 10 nM angiotensin II (Ang II) in normal (*Top*), pertussis-toxin-treated (*Middle*), and PMA-treated (1 μg/ml) (*Bottom*) cells. $[Ca^{2+}]_i$ was estimated as described (21).

When an analogous experiment was performed with hepatocytes isolated from rats treated with pertussis toxin, EGF was unable to increase $[Ca^{2+}]_i$ (Fig. 1 *Middle*) or to stimulate phosphorylase activity (not shown). In marked contrast, the response to angiotensin II appeared to be slightly exaggerated. The averaged data presented in Table 1 confirm that angiotensin II causes a slightly greater peak Ca^{2+} level in these cells without a change in the plateau level. In addition, prior treatment with toxin did not block the increase in hepatocyte Ca^{2+} caused by 20 nM vasopressin or 10 μM *l*-norepinephrine (data not shown).

The major effect of pertussis toxin on the plasma membrane appears to be ADP-ribosylation of the α subunit of N_i (25). The N_i extracted from the membranes of the intoxicated hepatocytes used in these experiments was demonstrated to be 100% ADP-ribosylated (n = 6) by using an assay that ensures complete ADP-ribosylation of the molecule in solution (19).

Phorbol esters have been demonstrated to inhibit the ability of EGF to bind to its receptor (1, 8) and to block the ability of EGF to stimulate the tyrosine kinase activity of the receptor (1, 9). When hepatocytes were treated with PMA for 3 min, the Ca^{2+} response to EGF was also abolished (Fig. 1 *Bottom*). In contrast, PMA did not affect the Ca^{2+} response to angiotensin II (Fig. 1 and Table 1).

Effects of EGF on the Phosphorylation of Cytosolic Proteins in Normal, Toxin-Treated, and PMA-Treated Hepatocytes. Previous studies have shown that there are distinct sets of substrates in the cytoplasm of hepatocytes that respond to the $InsP_3$ (Ca^{2+}) or $acyl_2Gro$ signals with marked increases in their phosphorylation state (23). Therefore, the ability of EGF and angiotensin II to stimulate protein phosphorylation in hepatocytes was examined to expose possible differences between the messengers generated by these two hormones. As expected, stimulation of ^{32}P-labeled hepatocytes with

Table 1. Hormone-stimulated changes in $[Ca^{2+}]_i$

Agonist and pretreatment	After stimulation: Time to peak, sec	Change in $[Ca^{2+}]_i$, fold/basal: At peak	Change in $[Ca^{2+}]_i$, fold/basal: On plateau
10 nM angiotensin II			
Normal cells	16.7 ± 5.1	8.4 ± 1.3	6.8 ± 1.5
+ toxin	23.0 ± 5.0	9.7 ± 2.0*	7.2 ± 1.0
+ PMA	21.5 ± 1.0	8.6 ± 1.4	5.2 ± 1.0
66 nM EGF			
Normal cells	45.6 ± 5.2†	3.8 ± 0.8‡	3.6 ± 0.7
+ toxin	43.9 ± 3.8	1.2 ± 0.1§	1.2 ± 0.1§
+ PMA	49.7 ± 4.1	1.2 ± 0.1§	1.2 ± 0.1§

Basal $[Ca^{2+}]_i$ measured 1–2 min prior to stimulation was 164–255 ± 59 nM and was unaffected by either pretreatment ($P < 0.05$). Plateau $[Ca^{2+}]_i$ was measured 2 min after stimulation. Toxin pretreatment was performed as described in *General Methods*. PMA pretreatment was performed as for Fig. 1. Values represent means ± SEM from four to seven experiments.

*Greater than in normal cells ($P < 0.05$).

†Longer than observed with angiotensin ($P < 0.001$).

‡Less than observed with angiotensin ($P < 0.05$).

§Different than EGF control ($P < 0.05$).

angiotensin II alters the phosphorylation state of 10 cytosolic proteins (Fig. 2 *Upper Right*, arrows pointing down). Spots a, b, and 29 represent proteins whose phosphorylation state is increased by phorbol esters or synthetic $acyl_2Gro$ analogues and are substrates for protein kinase C (23). Spots 4, 13, 17, 23, 34, 35, and 36 respond to increased levels of Ca^{2+} and are substrates for calmodulin-dependent protein kinases (23). When hepatocytes were stimulated with 66 nM EGF for 3 min, the phosphorylation state of these same 10 proteins was affected (Fig. 2 *Lower Left*, arrows pointing down). This result suggests that EGF may generate both the $acyl_2Gro$ and $InsP_3$ (Ca^{2+}) messages in hepatocytes.

However, EGF also stimulated the phosphorylation of an additional protein, spot c, with a molecular weight of 36,000 and an isoelectric point of 5.5 (Fig. 2 *Lower Left*, arrowhead pointing up). This event is not seen when cells are stimulated with glucagon or drugs such as the ionophore A23187, cyclic AMP, or phorbol esters. These results suggest that, in addition to generating $InsP_3$ and $acyl_2Gro$, EGF produces a novel phosphorylation event, possibly by activating an unknown protein kinase. Spot c was not resistant to hot KOH treatment (26), providing preliminary evidence that the unknown kinase is not a tyrosine kinase (data not shown).

The ability of EGF and angiotensin II to alter the phosphorylation state of the 11 cytoplasmic proteins was compared by quantitating the density information on the autoradiographs (22, 23). Three proteins whose phosphorylation is stimulated by angiotensin II, EGF, or both were selected for presentation in Table 2 because they represent the three intracellular messages under study. Spot 34 is markedly stimulated by increased Ca^{2+} (23), spot 29 is affected by $acyl_2Gro$ or phorbol esters (23), and spot c is the EGF-specific protein. Four other proteins (spots 10, 24, 27, and 33) represent controls ("benchmarks") whose phosphorylation state is not affected by hormones (Table 2) (22, 23). Note that, in normal hepatocytes, both angiotensin II and EGF markedly stimulate the phosphorylation of spots 29 and 34. However, in keeping with the effects of these two hormones on $[Ca^{2+}]_i$, angiotensin II appears to be more effective in stimulating the phosphorylation of these two proteins. In contrast, only EGF stimulates the phosphorylation of spot c.

When similar experiments were performed with hepatocytes isolated from toxin-treated rats, EGF was unable to stimulate the phosphorylation of the 10 substrates it shares with angiotensin II. However, the increase in phosphorylation of spot c (upward-pointing arrowhead) was still observed (compare *Lower* autoradiographs in Fig. 2). Toxin pretreatment had no effect on the ability of angiotensin II to stimulate protein phosphorylation in the cell (autoradiograph not shown). The quantitative data comparing the effects of the two hormones on the phosphorylation of the three selected proteins (messages) in intoxicated cells are shown in Table 2. Overall, these data suggest that ADP-ribosylation of N_i is able to block the generation of both the $acyl_2Gro$ and Ca^{2+} signals in response to EGF but not the unique message represented by spot c. Furthermore, modification of N_i does not affect the generation of $acyl_2Gro$ and Ca^{2+} ($InsP_3$) signals in response to angiotensin II. In fact, the phosphorylation of the two spots representing these messages (spots 29 and 34) appears to be enhanced in intoxicated cells (Table 2).

When normal hepatocytes were treated with PMA at 1 μg/ml for 8 min prior to stimulating the cells with EGF, the increase in phosphorylation of spots c and 34 was abolished (Fig. 3, Table 2). Fig. 3 presents enlargements of the lower right quadrants of the autoradiographs containing the three selected proteins (spots c, 29, and 34) as well as two benchmark proteins to allow comparison with Fig. 2. The data in Table 2 demonstrate that PMA has no effect on the ability of angiotensin II to generate the Ca^{2+} signal as monitored by changes in the phosphorylation of spot 34. Since PMA itself markedly stimulates the phosphorylation of spot 29 (ref. 23 and Fig. 3), no firm conclusions can be drawn regarding the ability of PMA to inhibit production of the $acyl_2Gro$ message in response to EGF. However, if PMA

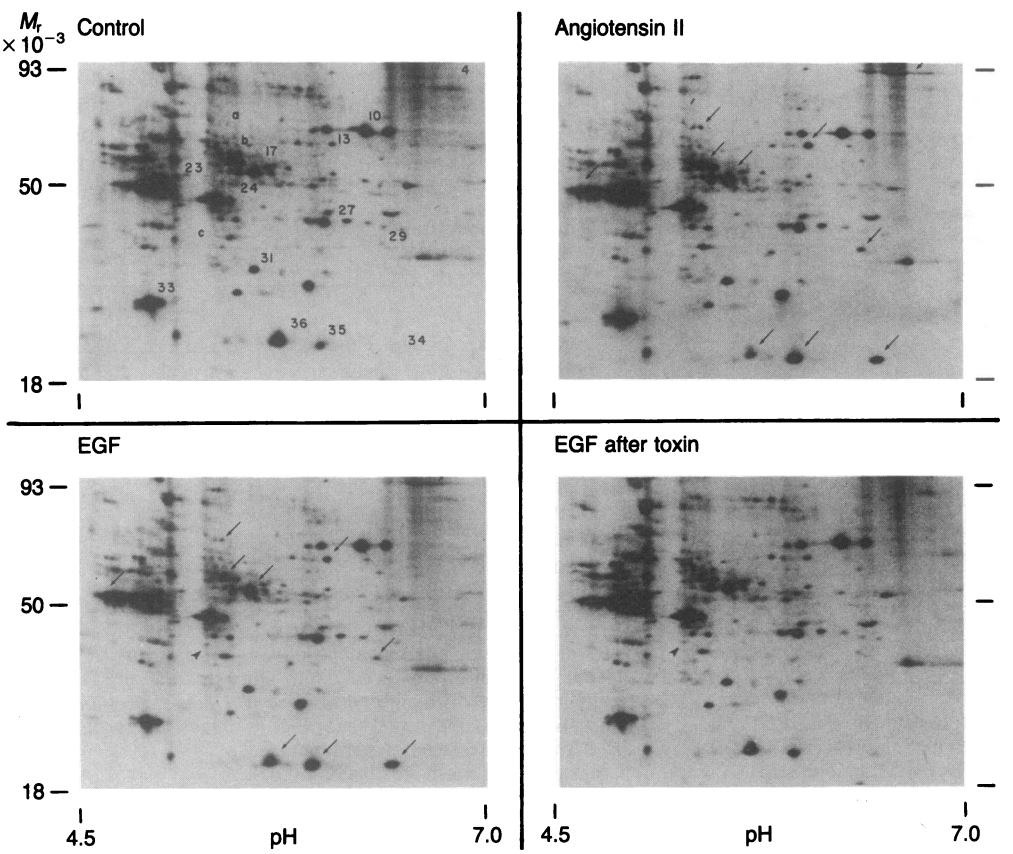

FIG. 2. Autoradiographs comparing the effects of EGF and angiotensin II on the phosphorylation state of cytosolic proteins in intact hepatocytes. ^{32}P-labeled cells were stimulated for 3 min with 10 nM angiotensin II (*Upper Right*) or 66 nM EGF (*Lower*) prior to preparation for electrophoresis. The numbers and letters in the control autoradiograph identifying each phosphoprotein are placed to the upper right of the spot. The numbering system is the same as used in previous publications (22, 23). The proteins whose phosphorylation state is altered by either angiotensin II or EGF treatment are identified by downward-pointing arrows. The product of the unique phosphorylation event generated by EGF, spot c, is identified by an upward-pointing arrowhead.

Table 2. Prior treatment with pertussis toxin or PMA alters the ability of EGF to stimulate protein phosphorylation

Protein (spot no.)	M_r	pI	Probable signal activating kinase	Change in phosphorylation, fold/control					
				Normal cells		Pretreatment: Pertussis toxin		Pretreatment: PMA	
				EGF	Ang II	EGF	Ang II	EGF	Ang II
Stimulated by hormone									
c	36,000	5.50	Unknown	6.1 ± 0.6	1.2 ± 0.1*	3.4 ± 0.1	0.95 ± 0.1*	0.97 ± 0.13*	0.96 ± 0.1*
29	35,000	6.70	acyl-$_2$Gro	5.6 ± 0.9	10.0 ± 2.7	1.1 ± 0.07*	13.4 ± 2.8	—†	—†
34	21,000	6.75	Ca^{2+}	3.2 ± 0.1‡	5.8 ± 1.0	0.8 ± 0.1*	20.9 ± 5.0	0.95 ± 0.1*	6.4 ± 0.9
Benchmarks									
10	67,000	6.45	—	1.1 ± 0.1	1.1 ± 0.07	1.0 ± 0.1	1.0 ± 0.09	0.98 ± 0.1	1.1 ± 0.1
24	42,000	5.60	—	1.03 ± 0.06	0.97 ± 0.03	0.9 ± 0.07	0.9 ± 0.07	0.94 ± 0.1	1.03 ± 0.1
27	38,000	6.15	—	1.1 ± 0.04	1.07 ± 0.05	1.0 ± 0.1	0.9 ± 0.02	1.0 ± 0.04	0.93 ± 0.1
33	25,000	4.87	—	1.0 ± 0.05	0.9 ± 0.04	0.9 ± 0.06	0.97 ± 0.04	0.97 ± 0.04	1.03 ± 0.1

^{32}P-labeled hepatocytes were treated with hormones as described in the legend to Fig. 2. Changes in phosphorylation state are reported as fold/control ± SEM from four to six experiments. Changes listed without footnotes are significantly different from the controls (benchmark proteins) ($P < 0.01$). PMA and pertussis toxin pretreatment were performed as described in *General Methods* and the legend to Fig. 3. Ang II, angiotensin II.

*Not different from benchmark proteins ($P < 0.5$).

†When PMA is used as a pretreatment, the effects of EGF and angiotensin II on the phosphorylation of spot 29 cannot be evaluated. PMA alone caused a 9-fold increase in phosphorylation of spot 29.

‡Less than angiotensin II ($P < 0.05$).

blocks the ability of EGF to generate the InsP_3 signal, it would be surprising if it did not inhibit production of acyl$_2$Gro, as the breakdown of each phosphatidylinositol 4,5-bisphosphate molecule is thought to yield both InsP_3 and acyl$_2$Gro (4).

DISCUSSION

Hormones such as angiotensin II stimulate the breakdown of phosphatidylinositol 4,5-bisphosphate in hepatocytes, generating InsP_3 (Ca^{2+}) and acyl$_2$Gro to act within the cell (4, 17). These messengers activate Ca^{2+}/calmodulin-dependent protein kinases and protein kinase C to generate the biochemical responses inherent to the hepatocyte (5, 6, 23). The measurements of intracellular Ca^{2+} levels and protein phosphorylation presented in this report suggest that EGF also generates the InsP_3 (Ca^{2+}) and acyl$_2$Gro signals in hepatocytes. However, the magnitude of the response to EGF is smaller than that generated by angiotensin II (Tables 1 and 2). It should be noted that direct measurements of InsP_3 and acyl$_2$Gro were not made during this study and thus the possibility remains that the changes observed may be due to other mechanisms.

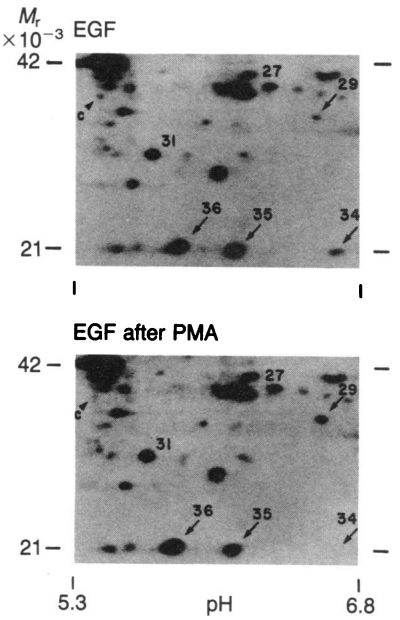

FIG. 3. Autoradiographs comparing the effect of EGF on the phosphorylation state of cytoplasmic proteins before and after PMA pretreatment. ^{32}P-labeled cells were stimulated for 3 min with 66 nM EGF before or after PMA pretreatment (1 μg/ml for 8 min). The spot identification system is the same as described in the legend to Fig. 2. Arrows and arrowheads depict the phosphoproteins whose phosphorylation state is changed by treatment of intact cells with EGF.

While both EGF and angiotensin II appear to stimulate inositol lipid breakdown in hepatocytes, there are major differences in the signal transduction mechanisms used by the two hormones to generate the response. The most striking differences are the ability of EGF to stimulate the phosphorylation of a unique substrate, spot c, and the finding that PMA and pertussis toxin abolish the InsP_3 and acyl$_2$Gro responses to EGF without affecting the generation of the same signals by angiotensin II.

Rats were treated with pertussis toxin for 72 hr in these experiments to obtain a complete modification of the N_i molecules in the hepatocyte membrane as judged by blockade of inhibitory inputs to adenylate cyclase and a sensitive ADP-ribosylation assay (ref. 19 and text). While it is possible that the stress of the treatment protocol blunted the response to EGF by an unknown mechanism, the intoxicated hepatocytes respond normally to glucagon (19) and angiotensin II or vasopressin (Figs. 1, 2 and text). Therefore, it seems likely that N_i or another toxin substrate such as G_o (27, 28) may couple the hepatic EGF receptor to the transduction system initiating the Ca^{2+} and protein phosphorylation events observed. Indeed, several laboratories have presented evidence that N_i or a similar protein may mediate the ability of various agonists to stimulate the inositol lipid response in mast cells (13), neutrophils (12, 14), and HL-60 cells (16). An important finding of the present experiments is that pertussis toxin did not block the signals produced by angiotensin II in hepatocytes. In fact, these responses may have been slightly enhanced as compared to normal cells (Tables 1 and 2). A somewhat analogous situation exists in 1321N1 astrocytoma cells, in which toxin does not block the phosphatidylinositol response to muscarinic agonists (29, 30). The overall implication of these results is that receptors can couple to the phospholipase C system via different transduction mechanisms. In the hepatocyte, EGF appears to utilize a pertussis toxin substrate such as N_i or G_o, whereas angiotensin II and

vasopressin do not. While current evidence suggests that angiotensin II receptors do not couple to phospholipase C via a pertussis toxin substrate, there is evidence to suggest that an unidentified guanine-nucleotide-binding protein may be involved in this type of phosphatidylinositol response (11, 19, 30).

Another difference between the responses of hepatocytes to EGF and angiotensin II is that EGF appears to stimulate the phosphorylation of a new substrate, spot c, with a molecular weight of 36,000. EGF might stimulate the phosphorylation of this protein by activating a protein kinase such as the ribosomal protein S6 kinase, which is thought to be stimulated by EGF and other growth factors (31, 32). An alternative explanation for the appearance of spot c could be the release of the protein from the membrane or another cellular compartment into the cytosol as a result of the EGF stimulus. It seems possible that the stimulus leading to the appearance of spot c is generated at a site in the transduction pathway prior to the activation of phospholipase C. This would explain why pertussis toxin could not abolish the EGF-stimulated phosphorylation of spot c (Fig. 2 and Table 2).

A third clear difference between the signal transduction pathways for angiotensin II and EGF is that PMA blocks all of the responses to EGF without altering those to angiotensin II (Fig. 3 and Table 2). It has been proposed that phorbol esters, apparently by activating protein kinase C (6), can exert feedback regulation on phosphatidylinositol metabolism (33). In addition, phorbol esters can decrease high-affinity binding of EGF to its receptor (1, 8) and decrease the tyrosine kinase activity of the EGF receptor (1, 9). The finding that PMA can block all responses to EGF in hepatocytes could certainly be explained by a protein kinase C-mediated effect on the EGF receptor. Alternatively, protein kinase C may phosphorylate a substrate involved in coupling the receptor to its transduction system. Thus EGF joins α_1-adrenergic agonists (34–36) and fMet-Leu-Phe (37) as agents whose effects on Ca^{2+} mobilization are blocked by pretreatment of the cells with phorbol esters. Apparently, protein kinase C does not exert the same effect on the angiotensin II or vasopressin (34–36) signal transduction system in hepatocytes.

While EGF appears to have the ability to stimulate inositol lipid breakdown in common with hormones such as angiotensin II or vasopressin, there are also major differences in the response of the cell to this hormone. It will be important to determine what role the phosphatidylinositol response plays in the overall series of cellular events initiated by this mitogen.

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1. Hunter, T. & Cooper, J. A. (1985) *Annu. Rev. Biochem.* **54**, 897–930.
2. Sawyer, S. T. & Cohen, S. (1981) *Biochemistry* **20**, 6280–6286.
3. Moolenaar, W. H., Tertoolen, L. G. J. & de Laat, S. W. (1984) *J. Biol. Chem.* **259**, 8066–8069.
4. Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360.
5. Schulman, H. (1982) in *Handbook of Experimental Pharmacology*, eds. Nathanson, J. A. & Kebabian, J. W. (Springer, New York), pp. 425–478.
6. Nishizuka, Y. (1984) *Nature (London)* **308**, 693–698.
7. Berridge, M. J. (1984) *Biotechnology* **2**, 541–546.
8. Magun, B. E., Matrisian, L. M. & Bowden, G. T. (1980) *J. Biol. Chem.* **255**, 6373–6381.
9. Cochet, C., Gill, G. N., Meisenhelder, J., Cooper, J. A. & Hunter, T. (1984) *J. Biol. Chem.* **259**, 2553–2558.
10. Gilman, A. G. (1984) *Cell* **36**, 577–579.
11. Cockcroft, S. & Gomperts, B. D. (1985) *Nature (London)* **314**, 534–536.
12. Bokoch, G. M. & Gilman, A. G. (1984) *Cell* **39**, 301–308.
13. Nakamura, T. & Ui, M. (1985) *J. Biol. Chem.* **260**, 3584–3593.
14. Volpi, M., Naccache, P. H., Molski, T. F. P., Shefcyk, J., Huang, C.-K., Marsh, M. L., Munoz, J., Becker, E. L. & Sha'afi, R. I. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2708–2712.
15. Murayama, T. & Ui, M. (1985) *J. Biol. Chem.* **260**, 7226–7233.
16. Brandt, S. J., Dougherty, R. W., Lapetina, E. G. & Niedel, J. E. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3277–3280.
17. Creba, J. A., Downes, C. P., Hawkins, P. T., Brewster, G., Michell, R. H. & Kirk, C. J. (1983) *Biochem. J.* **212**, 733–747.
18. Garrison, J. C. & Haynes, R. C., Jr. (1975) *J. Biol. Chem.* **250**, 2769–2777.
19. Pobiner, B. F., Hewlett, E. L. & Garrison, J. C. (1985) *J. Biol. Chem.* **260**, 16200–16209.
20. Charest, R., Blackmore, P. F., Berthon, B. & Exton, J. H. (1983) *J. Biol. Chem.* **258**, 8769–8773.
21. Tsien, R. Y., Pozzan, T. & Rink, T. J. (1982) *J. Cell Biol.* **94**, 325–334.
22. Garrison, J. C. & Wagner, J. D. (1982) *J. Biol. Chem.* **257**, 13135–13143.
23. Garrison, J. C., Johnsen, D. E. & Campanile, C. P. (1984) *J. Biol. Chem.* **259**, 3283–3292.
24. Williamson, J. R., Cooper, R. H., Joseph, S. K. & Thomas, A. P. (1985) *Am. J. Physiol.* **248**, C203–C216.
25. Ui, M. (1984) *Trends Pharmacol. Sci.* **5**, 277–279.
26. Cooper, J. A., Sefton, B. M. & Hunter, T. (1984) *Mol. Cell. Biol.* **4**, 30–37.
27. Sternweis, P. C. & Robishaw, J. D. (1984) *J. Biol. Chem.* **259**, 13806–13813.
28. Neer, E. J., Lok, J. M. & Wolf, L. G. (1984) *J. Biol. Chem.* **259**, 14222–14229.
29. Hughes, A. R., Martin, M. W. & Harden, T. K. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5680–5684.
30. Evans, T., Martin, M. W., Hughes, A. R. & Harden, T. K. (1985) *Mol. Pharmacol.* **27**, 32–37.
31. Novak-Hofer, I. & Thomas, G. (1984) *J. Biol. Chem.* **259**, 5995–6000.
32. Cobb, M. H. & Rosen, O. M. (1983) *J. Biol. Chem.* **258**, 12472–12481.
33. Orellana, S. A., Solski, P. A. & Brown, J. H. (1985) *J. Biol. Chem.* **260**, 5236–5239.
34. Lynch, C. J., Charest, R., Bocckino, S. B., Exton, J. H. & Blackmore, P. F. (1985) *J. Biol. Chem.* **260**, 2844–2851.
35. Cooper, R. H., Coll, K. E. & Williamson, J. R. (1985) *J. Biol. Chem.* **260**, 3281–3288.
36. Corvera, S., Schwartz, K. R., Graham, R. M. & García-Sáinz, J. A. (1986) *J. Biol. Chem.* **261**, 520–526.
37. Naccache, P. H., Molski, T. F. P., Borgeat, P., White, J. R. & Sha'afi, R. I. (1985) *J. Biol. Chem.* **260**, 2125–2131.