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Evidence that the epidermal growth factor receptor and non-tyrosine kinase hormone receptors stimulate phosphoinositide hydrolysis by independent pathways

John R. HEPLER,* Roger A. JEFFS,* William R. HUCKLE,†‡ Henry E. OUTLAW,§ Sue Goo RHEE,‖ H. Shelton EARP*†‡ and T. Kendall HARDEN*¶

Departments of *Pharmacology and †Medicine, and ‡The Lineberger Cancer Research Centre, School of Medicine, University of North Carolina, Chapel Hill, NC 27599, §Department of Physical Sciences, Delta State University, Cleveland, MS 38733, and ‖Laboratory of Biochemistry, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20892, U.S.A.

We have shown previously that exposure of a non-transformed continuous line of rat liver epithelial (WB) cells to epidermal growth factor (EGF), adrenaline, angiotensin II or [Arg^8]vasopressin results in an accumulation of the inositol phosphates InsP_1, InsP_2 and InsP_3 [Hepler, Earp & Harden (1988) J. Biol. Chem. **263**, 7610–7619]. Studies were carried out with WB cells to determine whether the EGF receptor and other, non-tyrosine kinase, hormone receptors stimulate phosphoinositide hydrolysis by common, overlapping or separate pathways. The time courses for accumulation of inositol phosphates in response to angiotensin II and EGF were markedly different. Whereas angiotensin II stimulated a very rapid accumulation of inositol phosphates (maximal by 30 s), increases in the levels of inositol phosphates in response to EGF were measurable only following a 30 s lag period; maximal levels were attained by 7–8 min. Chelation of extracellular Ca^{2+} with EGTA did not modify this relative difference between angiotensin II and EGF in the time required to attain maximal phospholipase C activation. Under experimental conditions in which agonist-induced desensitization no longer occurred in these cells, the inositol phosphate responses to EGF and angiotensin II were additive, whereas those to angiotensin II and [Arg^8]vasopressin were not additive. In crude WB lysates, angiotensin II, [Arg^8]vasopressin and adrenaline each stimulated inositol phosphate formation in a guanine-nucleotide-dependent manner. In contrast, EGF failed to stimulate inositol phosphate formation in WB lysates in the presence or absence of guanosine 5′-[γ-thio]triphosphate (GTP[S]), even though EGF retained the capacity to bind to and stimulate tyrosine phosphorylation of its own receptor. Pertussis toxin, at concentrations that fully ADP-ribosylate and functionally inactivate the inhibitory guanine-nucleotide regulatory protein of adenylate cyclase (G_i), had no effect on the capacity of EGF or hormones to stimulate inositol phosphate accumulation. In intact WB cells, the capacity of EGF, but not angiotensin II, to stimulate inositol phosphate accumulation was correlated with its capacity to stimulate tyrosine phosphorylation of the 148 kDa isoenzyme of phospholipase C. Taken together, these findings suggest that, whereas angiotensin II, [Arg^8]vasopressin and α_1-adrenergic receptors are linked to activation of one or more phospholipase(s) C by an unidentified G-protein(s), the EGF receptor stimulates phosphoinositide hydrolysis by a different pathway, perhaps as a result of its capacity to stimulate tyrosine phosphorylation of phospholipase C-γ.

INTRODUCTION

The physiological actions of a large class of hormones and neurotransmitters can be explained, in part, by their capacity to activate plasma-membrane-associated phosphoinositidases (i.e. phospholipase C) in target tissues (Downes & Michell, 1985; Berridge, 1987). Activation of phospholipase C by cell surface receptors results in hydrolysis of phosphatidylinositol 4,5-bisphosphate to form two second messengers, Ins(1,4,5)P_3 and *sn*-1,2-diacylglycerol. Ins(1,4,5)P_3 releases Ca^{2+} from intracellular stores, whereas diacylglycerol activates protein kinase C; both cytosolic Ca^{2+} and protein kinase C now are recognized to be important regulators of cell function (Kikkawa & Nishizuka, 1986; Berridge, 1987). More recently, it has been demonstrated that activation of receptors for mitogenic peptides including epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) also results in stimulation of phospholipase C activity in target tissues (Berridge *et al.*, 1984; Pike & Eakes, 1987; Hepler *et al.*, 1987; Johnson & Garrison, 1987; Tilly *et al.*, 1988; Tilly & Moolenaar, 1989; Wahl *et al.*, 1988).

Although recent findings have contributed significantly to the understanding of the biochemical events that occur subsequent to hormone-stimulated formation of Ins(1,4,5)P_3 and diacylglycerol, much less is known about the events that underlie receptor–effector coupling and activation of phospholipase C. Increasing evidence strongly suggests that many receptors for hormones and neurotransmitters are linked to activation of phospholipase C by one or more as yet unidentified guanine-nucleotide-binding regulatory proteins (G-proteins) (Litosch *et al.*, 1985; Cockcroft & Gomperts, 1985; Martin, 1989; Harden, 1989). In contrast, the mechanism whereby stimulation of growth factor receptors serves to regulate phospholipase C activity is not known, although this topic has been the subject of intense study (Paris *et al.*, 1988; Blakeley *et al.*, 1989; Tilly & Moolenaar, 1989). Receptors for EGF and PDGF are different from other hormone receptors linked to phosphoinositide hydrolysis in that they possess intrinsic tyrosine-specific protein kinase activity (Carpenter, 1987; Yarden & Ullrich, 1988) and share little or no amino acid sequence similarity or predicted structure with the closely related 'superfamily' of G-protein-linked receptors

Abbreviations used: EGF, epidermal growth factor; PDGF, platelet-derived growth factor; G-protein, guanine-nucleotide-binding regulatory protein; GTP[S], guanosine 5′-[γ-thio]triphosphate; GDP[S], guanosine 5′-[β-thio]diphosphate; Gpp[NH]p, guanosine 5′-[βγ-imido]triphosphate; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride.

¶ To whom all correspondence should be addressed.

(O'Dowd *et al.*, 1989). Previous findings are consistent with the idea that PDGF may stimulate phosphoinositide hydrolysis in fibroblasts by a mechanism that is different from that utilized by hormones such as bombesin (Blakeley *et al.*, 1989; Cattaneo & Vincentini, 1989). Furthermore, recent reports demonstrate that a 148 kDa isoenzyme of phospholipase C, i.e. phospholipase C-γ (Rhee *et al.*, 1989), serves as a substrate *in vitro* as well as *in vivo* for the tyrosine kinase activity associated with both the EGF and the PDGF receptors (Wahl *et al.*, 1989*a*,*b*; Nishibe *et al.*, 1989; Meisenhelder *et al.*, 1989). These observations raise the possibility that receptors for EGF may stimulate phosphoinositide hydrolysis by a mechanism that is different from that of other hormone receptors. To test this idea, studies were carried out on a non-transformed continuous line of rat liver epithelial cells (WB cells) to determine whether EGF and other hormones stimulate phosphoinositide hydrolysis by common, overlapping or independent pathways. We report findings consistent with the idea that EGF and other hormones activate phospholipase C by independent pathways.

MATERIALS AND METHODS

Materials

EGF was prepared from mouse submaxillary glands as previously described (Savage & Cohen, 1972). Angiotensin II, [Arg^8]vasopressin and GTP[S] were obtained from Boehringer Mannheim; adrenaline phorbol 12-myristate 13-acetate and forskolin were obtained from Sigma. *myo*-[^{3}H]Inositol (15 Ci/mmol) was obtained from American Radiolabelled Chemical Co. (St. Louis, MO, U.S.A.). Pertussis toxin was purchased from List Biologicals. Anti-(phospholipase C-γ) antibodies were prepared as previously described (Suh *et al.*, 1988).

Cell culture

WB cells were grown as previously described (Tsao *et al.*, 1984) with minor modifications (Hepler *et al.*, 1988). Briefly, WB stocks were maintained at 37 °C in an 8% CO_2 humidified atmosphere in Richter's improved minimal essential medium containing insulin (4 mg/ml) and L-glutamine (Irvine Scientific, Santa Ana, CA, U.S.A.) supplemented with 5% fetal calf serum and antibiotics. All stock plates were confluent and 5–7 days old at the time of subculture. Subculture was accomplished by aspirating the medium and detaching the cells by addition of 1 ml of trypsin (0.25%) in iso-osmotic citrate buffer (pH 7.4) for 15–30 min. Cells were seeded at 1:50 dilution, and for most experiments cells were routinely grown on 6-well plastic culture dishes (Costar).

Measurement of inositol phosphates in intact WB cells

WB cells were labelled with *myo*-[^{3}H]inositol and assayed for stimulated accumulation of [^{3}H]inositol phosphates as described previously (Hepler *et al.*, 1988).

Measurement of inositol phosphates in WB lysates

WB cells were rinsed once with iso-osmotic buffer (0.9% NaCl, 1 mM-EDTA, 10 mM-Hepes, pH 7.0) and were then collected at 4 °C in the same buffer containing a cocktail of proteinase inhibitors including leupeptin (0.5 μg/ml), pepstatin A (0.7 μg/ml), aprotinin (0.5 μg/ml), phenylmethanesulphonyl fluoride (PMSF) (0.1 mM) and benzamidine (0.1 mM); PMSF was freshly prepared in propan-2-ol and added to the lysis buffer immediately before preparation of lysates. Cell suspensions were homogenized by ten strokes in a ground glass homogenizer at 4 °C. Under these conditions more than 90% of the WB cells were lysed as determined by Trypan Blue exclusion under microscopic examination. Assays were initiated by addition of 50 μl of lysates to an intracellular-like assay buffer (Harden *et al.*, 1987); the final assay volume was 200 μl. Incubations were carried out at 37 °C and the reactions were stopped by addition of 0.5 ml of ice-cold $HClO_4$ (6.25%, v/v). The acid-inactivated samples were centrifuged for 5 min, and supernatants were collected and neutralized (Harden *et al.*, 1987). Measurement of [^{3}H]inositol phosphates was achieved by anion-exchange chromatography as described above.

Measurement of adenylate cyclase activity

Assays to measure guanine-nucleotide-mediated inhibition of forskolin-stimulated adenylate cyclase activity in broken cell preparations were performed as previously described (Harden *et al.*, 1982).

Measurement of pertussis toxin-catalysed ADP-ribosylation of WB membranes

WB cells were pretreated overnight with normal culture medium in either the presence or the absence of the indicated concentrations of pertussis toxin (0–300 ng/ml). Washed membranes were prepared from control and toxin-treated cells. ADP-ribosylation reactions, SDS/PAGE and autoradiography were carried out as previously described (Martin *et al.*, 1985).

Immunoprecipitation and measurement of tyrosine phosphorylation of phospholipase C

WB cells were grown in 60 mm diameter plastic dishes. Following treatment with hormone or growth factor, incubations were terminated by rapid aspiration of the medium and addition (1 ml per dish) of ice-cold lysis buffer containing 20 mM-NaHepes, 50 mM-NaF, 10% (v/v) glycerol, 1% (w/v) Triton X-100, 1 mM-sodium orthovanadate, 10 μg of PMSF/ml and 2.5 mM-*p*-nitrophenylphosphate (Wahl *et al.*, 1989*a*). Lysates were clarified by centrifugation (12000 *g*; 10 min; 4 °C). Phospholipase C-γ was immunoprecipitated by incubation with monoclonal anti-(phospholipase C-γ) antibodies (0.5 μg/tube), 5 μg of rabbit anti-(mouse IgG) and 10 μl of Pansorbin (Calbiochem). After 4 h at 4 °C, immunoprecipitates were collected by centrifugation (12000 *g*, 10 min) and the pellets were washed once (1 ml/tube) with lysis buffer. For assays using WB lysates, cells were first prepared as lysates as described above and were treated with vehicle, angiotensin II or EGF in the presence or absence of GTP[S] following the same protocol and using the same assay buffers as described above for measuring inositol phosphate formation in WB lysates. Reactions were terminated by addition of 900 μl of lysis buffer to 200 μl of reaction mix. Solubilized extracts then were clarified and immunoprecipitated as described above. This procedure was found to precipitate all detectable (i.e. immunoreactive) phospholipase C-γ from WB cell extracts. Immunoprecipitated proteins were solubilized for SDS/PAGE on 7% polyacrylamide gels, transferred to nitrocellulose membranes and then subjected to anti-phosphotyrosine immunoblotting and autoradiography as described (McCune & Earp, 1989). [*P*-Tyr]Phospholipase C-γ was located on immunoblots by its apparent molecular mass and by co-migration with the unique immunoreactive species ($\sim$ 150 kDa) identified by immunoblotting with monospecific anti-(phospholipase C-γ) antibodies. Evidence that the 170–180 kDa band is the EGF receptor was provided by the fact that pre-clearing of the solubilized cell lysates with anti-(EGF receptor) antisera (ab 1382) before immunoprecipitation with anti-phospholipase-γ) antibodies resulted in a selective loss of the 170 kDa band (Huckle *et al.*, 1990).

Statistical analysis

Statistical analysis of the data was performed by Student's *t* test.

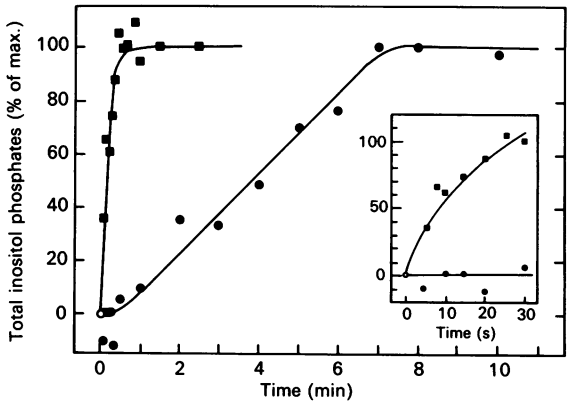

Fig. 1. Time course for accumulation of inositol phosphates in response to angiotensin II (■) or EGF (●)

WB cells were labelled overnight with 10 μCi of [^{3}H]inositol/ml in inositol-free medium containing 5% fetal calf serum. Following labelling, cells were treated with 300 ng of EGF/ml (●) or 1 μM-angiotensin II (■) for the indicated times at 37 °C in the presence of 10 mM-LiCl. Following termination of the reaction, samples were assayed for the accumulation of [^{3}H]inositol phosphates by anion-exchange chromatography. For comparative purposes, the data are presented as the percentage of accumulation at the earliest time of maximal response for angiotensin II (i.e. 30 s) and EGF (i.e. 7 min). The maximal EGF response typically represented 40–60% of the maximal angiotensin II response. The data are pooled results from four and three separate experiments with EGF and angiotensin II respectively.

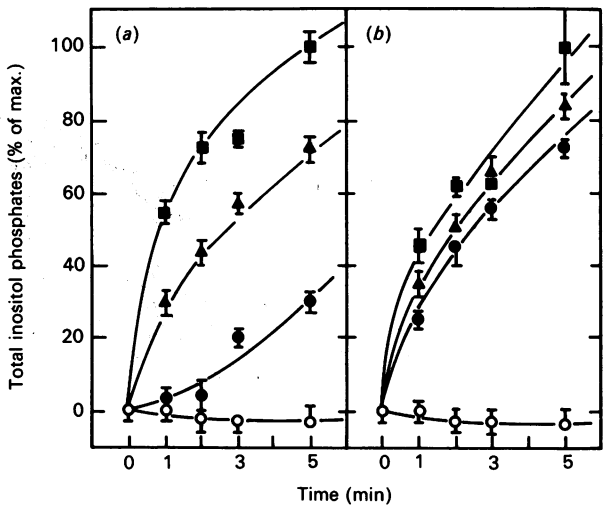

Fig. 2. Additivity of the inositol phosphate response to EGF and hormones

WB cells were incubated overnight with 10 μM-PMA and 10 μCi of [^{3}H]inositol/ml in inositol-free medium containing 5% fetal calf serum. Following labelling, cells were treated as follows. (*a*) Vehicle (○), EGF (300 ng/ml) (●), 1 μM-angiotensin II (▲), or angiotensin II plus EGF (■); (*b*) vehicle (○), angiotensin II (▲), [Arg^8]-vasopressin (●), or angiotensin II plus [Arg^8]vasopressin (■) for the indicated times at 37 °C in the presence of 10 mM-LiCl. On termination of the reaction, samples were assayed for the accumulation of [^{3}H]inositol phosphates by anion-exchange chromatography. The maximal responses to EGF + angiotensin II (9810 ± 448 c.p.m.; *a*) and to angiotensin II + [Arg^8]vasopressin (8719 ± 870 c.p.m., *b*) were defined as 100%. The data are the means ± S.E.M. of triplicate determinations and are representative of three experiments.

RESULTS

We have previously demonstrated that exposure of intact WB cells to EGF, adrenaline, angiotensin II or [Arg^8]vasopressin results in an increase in the cellular levels of InsP_1, InsP_2 and InsP_3 (Hepler *et al.*, 1988; Earp *et al.*, 1988). Experiments were carried out to determine the comparative time courses for accumulation of inositol phosphates in response to EGF or angiotensin II in WB cells. Consistent with previous findings (Hepler *et al.*, 1988), angiotensin II stimulated a very rapid accumulation of inositol phosphates that reached a maximum within 30 s (Fig. 1). The rapid termination of this response is apparently due to a protein kinase C-mediated agonist-induced desensitization of the receptor-regulated phospholipase C (Hepler *et al.*, 1988). In contrast, the rate of accumulation of inositol phosphates in response to EGF was considerably slower than that for angiotensin II (Fig. 1). After addition of EGF, a brief lag period (approx. 30 s) was observed before increased levels of inositol phosphates were detected (Fig. 1, inset). Inositol phosphates then continued to accumulate until maximal levels were attained by approx. 7–8 min. Using h.p.l.c. analysis, we have shown previously that exposure to angiotensin II stimulates the rapid, albeit transient, accumulation of Ins(1,4,5)P_3 and Ins(1,3,4)P_3 in WB cells (Hepler *et al.*, 1988). EGF also stimulates an accumulation of metabolic products of Ins(1,4,5)P_3 in WB cells, although increases in radioactive products only occur after periods of growth factor exposure greater than 30 s (results not shown). The relative difference in the time courses of inositol phosphate accumulation in response to angiotensin II and EGF was not abolished by chelation of extracellular Ca^{2+}, although in three experiments EGTA attenuated (0–30% inhibition) the maximal level of inositol phosphate accumulation observed (results not shown).

Experiments were carried out to test whether the inositol phosphate responses to maximally effective concentrations of EGF and angiotensin II were additive. A meaningful interpretation of data obtained from additivity studies would not be possible under conditions in which agonist-induced desensitization occurs. This fact presented a problem in WB cells, since we have previously shown that the inositol phosphate response to angiotensin II or [Arg^8]vasopressin desensitizes rapidly. However, in cells pretreated overnight with PMA, protein kinase C is down-regulated, and agonist-induced desensitization of the inositol phosphate response to angiotensin II and [Arg^8]vasopressin no longer occurs (Hepler *et al.*, 1988). Therefore, to determine whether accumulation of inositol phosphates in response to maximally effective concentrations of EGF, angiotensin II and [Arg^8]vasopressin was additive, experiments were carried out in cells pretreated overnight with PMA. EGF and angiotensin II each stimulated inositol phosphate accumulation, although the EGF response was smaller and occurred much more slowly than that for angiotensin II (Fig. 2*a*). When EGF and angiotensin II were added simultaneously, inositol phosphates accumulated to levels equal to or greater than the sum of the responses observed for angiotensin II and EGF alone. In contrast to EGF, [Arg^8]vasopressin stimulated an accumulation of inositol phosphates that followed a time course and reached levels comparable with those stimulated by angiotensin II alone (Fig. 2*b*). Furthermore, simultaneous addition of these two hormones resulted in only a small (non-additive) increase in the accumulated levels of inositol phosphates observed in cells treated with either hormone alone. Additional studies were carried out to determine whether the inositol phosphate responses to EGF, angiotensin II and [Arg^8]vasopressin were additive under different experimental conditions. PKC-depleted WB cells were treated first with a maximally effective concentration of angiotensin II (1 μM) for 5–20 min, followed by the further addition of angiotensin II,

Table 1. Guanine nucleotide-dependent stimulation of inositol phosphate production by hormones but not by EGF in WB cell lysates

Lysates were prepared from [^{3}H]inositol-prelabelled WB cells as described in the Materials and methods section. Lysates were incubated at 37 °C for 10 min in the presence of vehicle, the indicated concentrations of hormone, EGF or GTP[S], or hormone or EGF in the presence of GTP[S]. Following termination of the reaction, samples were assayed for the presence of [^{3}H]inositol phosphates. All assays were carried out in the presence of 10 mM-LiCl and 300 μM-GDP[S]. The results are presented as the means ± S.E.M. of quadruplicate determinations and are representative of two experiments. Statistical significance is indicated as follows. * The increase in radioactivity due to stimulation by agonist and GTP[S] is significantly greater than that due to stimulation by GTP[S] alone ($P < 0.01$). † The increase in radioactivity due strictly to agonist in the presence of GTP[S] [(agonist + GTP[S]) − GTP[S]] is significantly greater ($P < 0.01$) than that due to agonist alone (agonist − vehicle).

Treatment	Inositol phosphates (c.p.m.)
Vehicle	96 ± 26
EGF (300 ng/ml)	159 ± 19
Adrenaline (10 μM)	140 ± 47
Angiotensin II (1 μM)	189 ± 30
[Arg^8]Vasopressin (1 μM)	367 ± 18
GTP[S] (100 μM)	887 ± 44
GTP[S] (100 μM) + EGF (300 ng/ml)	880 ± 25
GTP[S] (100 μM) + adrenaline (10 μM)	1184 ± 74*†
GTP[S] (100 μM) + angiotensin II (1 μM)	1950 ± 66*†
GTP[S] (100 μM) + [Arg^8]vasopressin (1 μM)	1966 ± 48*†

EGF (300 ng/ml) or [Arg^8]vasopressin (1 μM). Under these conditions, addition of either angiotensin II or [Arg^8]vasopressin did not stimulate a further increase in the levels of inositol phosphates. In contrast, EGF addition resulted in an increase (additive with that observed with angiotensin II alone) in the levels of inositol phosphates identical with that observed in control cells (results not shown).

Many receptors for hormones and neurotransmitters activate phospholipase C in a guanine-nucleotide-dependent manner, suggesting the involvement of a G-protein in the phosphoinositide signalling pathway (Martin, 1989; Harden, 1989). Experiments were carried out to determine the role of guanine nucleotides in the regulation of EGF- and/or hormone-stimulated activation of phospholipase C in lysates prepared from [^{3}H]inositol-prelabelled WB cells. Since it is possible that EGF may activate phospholipase C by a mechanism requiring the presence of unknown cytosolic factors, crude lysates prepared with a cocktail of proteinase inhibitors were used for these experiments. To test the absolute dependency of the hormone- and EGF-stimulated responses on exogenously added guanine nucleotides, all assays were carried out in the presence of guanosine 5′-[β-thio]diphosphate (GDP[S]) to block the actions of endogenous GTP. EGF, adrenaline, angiotensin II and [Arg^8]vasopressin each stimulated little or no accumulation of inositol phosphates when added alone (Table 1). In contrast, addition of GTP[S] (a non-hydrolysable analogue of GTP) resulted in a large increase in the levels of inositol phosphates: in the presence of GTP[S], adrenaline, angiotensin II and [Arg^8]vasopressin, but not EGF, caused an increase in inositol phosphate production greater than that observed with GTP[S] alone. As illustrated in Fig. 3, EGF and adrenaline each stimulated a concentration-dependent accumulation of inositol phosphates in intact WB cells. However, in crude lysates, neither EGF nor adrenaline alone stimulated inositol phosphate formation at all concentrations tested; in the

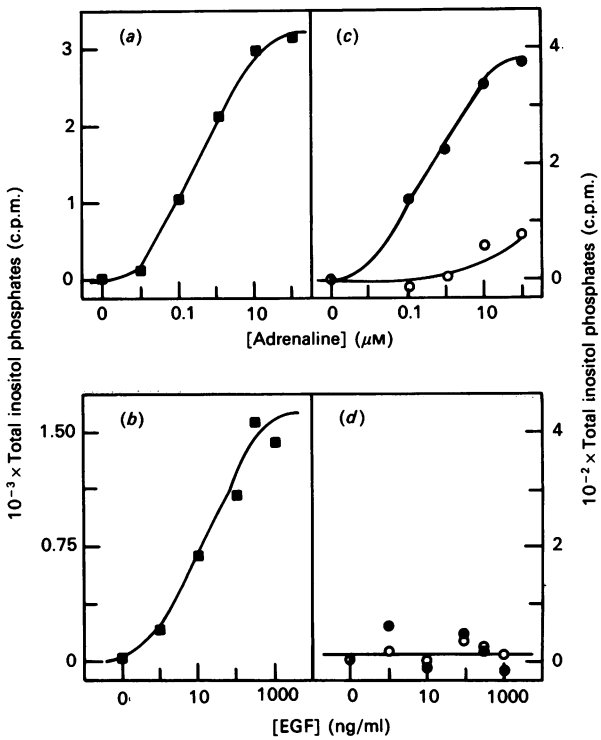

Fig. 3. Differential stimulation of inositol phosphate accumulation by adrenaline and EGF in intact WB cells or WB cell lysates

WB cells were labelled overnight with [^{3}H]inositol in inositol-free medium containing 5% fetal calf serum. After labelling, cells were treated with the indicated concentrations of adrenaline (*a*) or EGF (*b*) for 10 min in the presence of 10 mM-LiCl. On termination of the reaction, cells were assayed for [^{3}H]inositol phosphates by anion-exchange chromatography. In separate experiments, [^{3}H]inositol-labelled WB cells were prepared as crude lysates as described in the Materials and methods section. Lysates were incubated with the indicated concentrations of adrenaline (*c*) or EGF (*d*) in the presence (●) or absence (○) of 100 μM-GTP[S] for 10 min at 37 °C in assay buffer containing 300 μM-GDP[S] and 10 mM-LiCl. On termination of the reaction, cells were assayed for [^{3}H]inositol phosphates by anion-exchange chromatography. To more clearly compare the inositol phosphate responses due to adrenaline and EGF in the absence or presence of guanine nucleotide, the increase in [^{3}H]inositol phosphate due to GTP[S] alone was subtracted from the values presented.

presence of GTP[S], adrenaline but not EGF was capable of stimulating inositol phosphate formation in a concentration-dependent manner. In addition, in digitonin-permeabilized WB cells, adrenaline and angiotensin II, but not EGF, were capable of stimulating phospholipase C activity in a guanine nucleotide-dependent manner (results not shown). Using incubation conditions identical with those used for observation of guanine nucleotide-dependent formation of inositol phosphates by hormones, EGF was capable of binding to and stimulating the autophosphorylation of its own receptor in both lysates and permeabilized cells (results not shown). These findings suggest that the lack of capacity of EGF to activate phospholipase C in cell-free preparations cannot be explained by a lack of capacity of EGF to bind to and activate its own receptor.

Johnson & Garrison (1987) reported that pertussis toxin blocked the accumulation of Ins(1,4,5)P_3 and the mobilization of intracellular Ca^{2+} in response to EGF, but not angiotensin II, in primary cultures of rat hepatocytes. Overnight pretreatment of WB cells with concentrations of pertussis toxin up to 1000 ng/ml did not alter the inositol phosphate response either to EGF

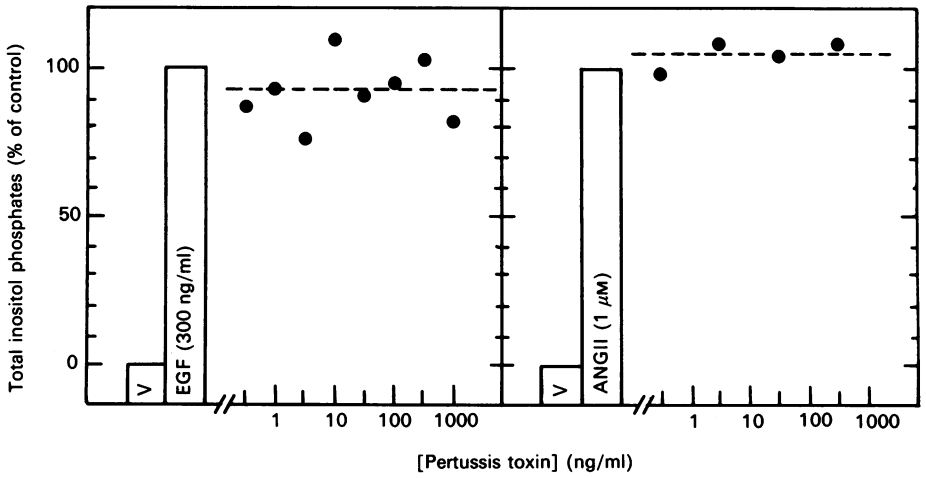

Fig. 4. Lack of effect of pertussis toxin treatment on EGF- or angiotensin II-stimulated inositol phosphate accumulation in WB cells

WB cells were labelled overnight with [^{3}H]inositol in inositol-free medium containing 5% fetal calf serum in the presence or absence of the indicated concentrations of pertussis toxin. Following labelling, cells were treated with vehicle (V) or 300 ng of EGF/ml or 1 μM-angiotensin II (ANG II) for 10 min at 37 °C in the presence of 10 mM-LiCl. On termination of the reaction, samples were assayed for the accumulation of [^{3}H]inositol phosphate by anion-exchange chromatography. The data are pooled results from two experiments performed in triplicate.

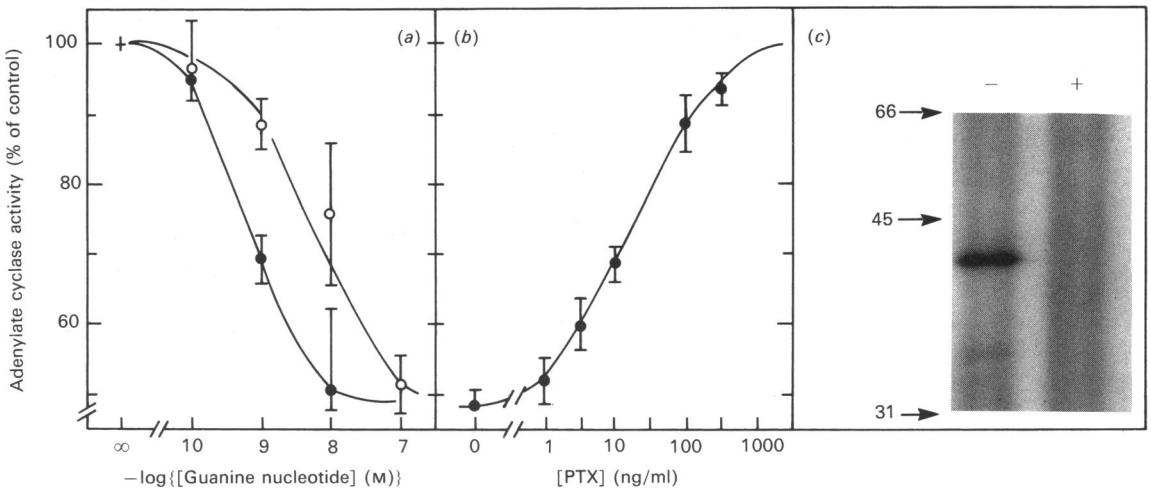

Fig. 5. Pertussis toxin-stimulated [^{32}P]ADP-ribosylation of G_i and blockade of guanine nucleotide-mediated inhibition of adenylate cyclase activity

(*a*) Membranes were prepared from WB cells and treated with 100 μM-forskolin in the absence or presence of the indicated concentrations of GTP[S] (●) or Gpp[NH]p (○) for 10 min at 30 °C. Results are plotted as the percentage of forskolin-stimulated adenylate cyclase activity in the absence of guanine nucleotide and are representative of two experiments. (*b*) WB cells were treated overnight with the indicated concentrations of pertussis toxin (PTX). Membranes were prepared and adenylate cyclase assays were carried out in the presence of forskolin (100 μM) or forskolin plus GTP[S] (10 μM). The results are plotted as the percentage of adenylate cyclase activity in the absence of GTP[S] and are representative of two experiments. (*c*) Plasma membranes were prepared from cells treated overnight with either vehicle (−) or 300 ng of pertussis toxin/ml (+). Membranes then were treated with pertussis toxin and [^{32}P]NAD$^+$ and subjected to SDS/PAGE as described in the Materials and methods section. An autoradiogram showing molecular masses (kDa) of protein standards is presented.

(Fig. 4*a*) or to angiotensin II (Fig. 4*b*). Pertussis toxin treatment also failed to abolish or attenuate the inositol phosphate responses to adrenaline and [Arg^8]vasopressin (results not shown). Nevertheless, WB cells apparently do express a functional G_i protein, since GTP[S] and guanosine 5′-[βγ-imido]triphosphate (Gpp[NH]p) each inhibited forskolin-stimulated adenylate cyclase activity in a concentration-dependent manner (Fig. 5*a*). The inability of pertussis toxin to alter hormone- or growth factor-stimulated inositol phosphate response occurred under conditions in which the toxin completely abolished the guanine nucleotide-mediated inhibition of forskolin-stimulated adenylate cyclase activity (Fig. 5*b*), and where there was apparently complete ADP-ribosylation of a 41 kDa membrane protein, presumably the α-subunit(s) of G_i (Fig. 5*c*).

It previously has been demonstrated that EGF stimulates tyrosine phosphorylation of an approx. 150 kDa protein identified as an isoenzyme of phospholipase C (i.e. phospholipase C-γ) in A431 human epidermoid carcinoma cells (Wahl *et al.*, 1989*a*; Nishibe *et al.*, 1989). The effects of EGF and angiotensin II on the phosphorylation of phospholipase C-γ was examined in WB cells. Cells were treated with EGF or angiotensin II for

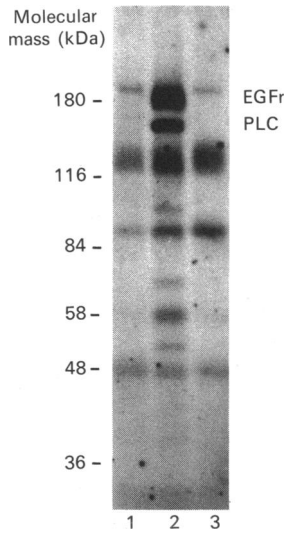

Fig. 6. EGF-stimulated tyrosine phosphorylation of phospholipase C-γ in WB cells

WB cells were treated with vehicle (lane 1), 300 ng of EGF/ml (lane 2), or 1 μM-angiotensin II (lane 3) for 1 min at 37 °C in Hepes-buffered Dulbecco's modified Eagle's medium (pH 7.4) as described in the Materials and methods section. Incubations then were terminated, cells were collected and samples were solubilized and immunoprecipitated with anti-(phospholipase C-γ) antibodies as described. Immunoprecipitates were subjected to SDS/PAGE, transblotted to nitrocellulose, screened for anti-phosphotyrosine immunoreactivity and visualized using ^{125}I-labelled Protein A and autoradiography. The mobilities of the EGF receptor (EGFr) and phospholipase C-γ (PLC) are indicated.

1 min and then lysed, solubilized and immunoprecipitated with anti-(phospholipase C-γ) antibodies (Suh *et al.*, 1988). Immunoprecipitates were washed, subjected to SDS/PAGE, transblotted to nitrocellulose and then screened for phosphotyrosine immunoreactivity. EGF markedly stimulated tyrosine phosphorylation of several proteins, including species with properties (see the Materials and methods section) consistent with those of the ~ 150 kDa phospholipase C-γ and the 170 kDa EGF receptor. In contrast with the effects of EGF on phosphorylation of phospholipase C-γ and the EGF receptor, angiotensin II did not stimulate tyrosine phosphorylation of phospholipase C-γ during a 1 min incubation (Fig. 6) or with longer incubation times (results not shown). However, both EGF and angiotensin II stimulated tyrosine phosphorylation of an unidentified 93 kDa protein (Fig. 6).

DISCUSSION

The results presented here demonstrate that EGF and angiotensin II stimulate inositol phosphate accumulation in WB cells at markedly different rates. The lag associated with appearance of the EGF response and the clear differences in the rates of accumulation of inositol phosphates stimulated by EGF and angiotensin II lend support to the idea that EGF stimulates phosphoinositide hydrolysis by a pathway distinct from that utilized by classical hormones. This idea is further supported by the observation that the inositol phosphate responses to EGF and angiotensin II are additive whereas the responses to angiotensin II and [Arg^8]vasopressin are not. The more rapid rate of accumulation of inositol phosphates in response to angiotensin II suggests a direct link to activation of phospholipase C, as might be expected for a hormone receptor that interacts with a G-protein-activated phospholipase C (Boyer *et al.*, 1989). In contrast, the lag in activation and the much slower rate of accumulation of inositol phosphates characteristic of the EGF response could reflect any of a number of possibilities. For example, multiple steps could exist between receptor activation and increased hydrolysis of phosphoinositides.

Cell surface receptors for hormones and neurotransmitters are apparently coupled to activation of phospholipase C by one or more as yet unidentified G-proteins (Martin, 1989; Harden, 1989). Guanine-nucleotide-dependent activation of phospholipase C by adrenaline, angiotensin II and [Arg^8]vasopressin in broken-cell preparations from WB cells is consistent with this idea. In contrast, EGF failed to stimulate phospholipase C activity in either the presence or the absence of guanine nucleotides, although under these assay conditions EGF was fully capable of binding to and stimulating autophosphorylation of its receptor. Thus, if a G-protein is involved in EGF receptor action, it is not active under assay conditions that support hormone-receptor and guanine-nucleotide-stimulated activation of phospholipase C. Such a hypothetical G-protein either would be markedly different from the one involved in linking hormone receptors to activation of phospholipase C, or perhaps a factor(s) and/or ancillary protein required for EGF receptor–G-protein interaction may be non-functional in cell lysates. However, assay conditions were designed to minimize the latter possibility, and the simplest explanation is that a G-protein is not involved in EGF receptor action in WB cells. In support of this conclusion, several reports (Hasegawa-Sasaki *et al.*, 1988; Cattaneo & Vincentini, 1989) demonstrated that, in contrast to the situation with other hormones, PDGF-stimulated phosphoinositide hydrolysis in permeabilized fibroblasts is not dependent on the presence of added GTP or GTP analogues. Similarly, both studies showed that GDP[S] blocked the actions of hormones but not those of PDGF. However, Hasegawa-Sasaki *et al.* (1988) reported that GTP[S] apparently inhibited the PDGF response. These findings suggest that a possible role of a G-protein in PDGF action (albeit different from that associated with hormone receptors) cannot be completely ruled out. It should be noted, however, that Cattaneo & Vincentini (1989) failed to observe a GTP[S]-mediated inhibition of response to PDGF. The reasons for this discrepency remain unclear.

Other evidence suggests that the EGF receptor stimulates phospholipase C by a mechanism that does not involve a G-protein. Receptors for EGF share little or no amino acid sequence similarity or predicted structure with the superfamily of G-protein-linked hormone receptors (Carpenter, 1987; Yarden & Ullrich, 1988; O'Dowd *et al.*, 1989). Whether amino acid sequences exist in these growth factor receptors that impart the capacity to interact with G-protein(s) is not known, although no direct evidence exists to support this idea. Johnson & Garrison (1987) have reported that EGF-stimulated Ins(1,4,5)P_3 accumulation in rat hepatocytes is abolished by pertussis toxin pretreatment, suggesting the possible involvement of a G-protein in EGF receptor action in rat liver. However, pertussis toxin failed to alter either EGF- or hormone-stimulated phospholipase C activity in WB cells. This lack of pertussis toxin sensitivity occurred at concentrations of toxin that fully ADP-ribosylate and functionally inactivate G_i. It should be noted that WB cells are liver epithelial cells rather than the parenchymal hepatocytes studied by Johnson & Garrison (1987). Pike & Eakes (1987) also reported that EGF-stimulated inositol phosphate formation in A431 cells was not altered by pertussis toxin treatment, and thus it is possible that pertussis toxin blocks EGF-stimulated activation of phospholipase C in a cell-specific manner (Milligan, 1988; Martin, 1989). Alternatively pertussis toxin may act at sites other than receptor-coupled G-proteins in some cell types.

Consistent with this idea is the observation by Taylor *et al.* (1988) that pertussis toxin blocked bombesin-mediated events in Swiss 3T3 fibroblasts at an unidentified site distal to receptor–effector coupling.

Most isoenzymes of phospholipase C are activated, at least *in vitro*, by micromolar concentrations of Ca^{2+}, and EGF markedly elevates the cytoplasmic Ca^{2+} concentration in target tissues (Wheeler *et al.*, 1987; Hepler *et al.*, 1987; Wahl *et al.*, 1988). However, although chelation of extracellular Ca^{2+} attenuated the maximal accumulation of inositol phosphates in response to both EGF and hormones, it neither abolished the capacity of these agents to stimulate phosphoinositide hydrolysis nor altered the relative time to the maximal response. Thus, elevation of Ca^{2+} is not a primary mechanism whereby receptors for EGF (or hormones) stimulate activation of phospholipase C in WB cells. These findings are consistent with those of Tilly *et al.* (1988) who reported that the EGF-stimulated accumulation of inositol phosphates in A431 cells occurred more slowly than that caused by bradykinin, that the EGF response was additive to bradykinin stimulation, and that these responses did not require extracellular Ca^{2+}

Receptors for EGF are fundamentally different from G-protein-linked hormone receptors in another important respect, in that they express intrinsic tyrosine-specific protein kinase activity. What role, if any, the tyrosine kinase activity associated with the EGF receptor subserves in regulation of phospholipase C activity is not clear. Chen *et al.* (1987) and Moolenaar *et al.* (1988) demonstrated that inactivation of the EGF receptor kinase by site-directed mutagenesis abolished a variety of transmembrane signalling events associated with EGF receptor action, including the rapid release of cytoplasmic Ca^{2+} from intracellular stores [presumably mediated by Ins(1,4,5)P_3]. More recently, Wahl *et al.* (1988) have demonstrated that, following stimulation of EGF receptors on A431 cells, phospholipase C activity was recovered from soluble cell extracts by anti-phosphotyrosine antibody affinity chromatography. The phosphorylated phospholipase C was later identified as the ~ 150 kDa enzyme termed phospholipase C-γ by Rhee and co-workers (Ryu *et al.*, 1987; Rhee *et al.*, 1989), since monoclonal antibodies that recognize this phospholipase C (Suh *et al.*, 1988) precipitated tyrosine-phosphorylated protein from EGF-treated cells (Wahl *et al.*, 1989*a*). The present findings demonstrate that EGF-stimulated accumulation of inositol phosphates in intact WB cells is correlated with its capacity to stimulate the tyrosine phosphorylation of phospholipase C-γ, whereas the even more marked accumulation of inositol phosphates in the presence of angiotensin II occurs with no tyrosine phosphorylation of phospholipase C-γ. Thus there is no GTP-dependence of the effects of EGF in WB cells, and EGF-dependent regulation of phospholipase C occurs potentially as a result of receptor-stimulated tyrosine phosphorylation of phospholipase C-γ. Interestingly both EGF and angiotensin II stimulated phosphorylation of an approx. 93 kDa protein (Fig. 6; W. R. Huckle, unpublished work). Although the identity and function of this protein remain unknown, a possible role for this species, either direct or indirect, in the regulation of phospholipase C activity cannot be ruled out by the present data. The findings presented here with WB cells also demonstrate that, as is the case with A431 cells (Wahl *et al.*, 1989*a*), EGF receptors (and several other unidentified proteins) are co-precipitated by anti-(phospholipase C-γ) antibodies. These observations raise the possibility that these proteins may be functionally linked in a multi-component complex.

Although the complete amino acid sequences of at least five distinct proteins with phospholipase C-like activity have been reported (Rhee *et al.*, 1989), the identity of the isoenzyme(s) regulated by receptors and G-proteins is not yet known. EGF receptors stimulate tyrosine phosphorylation of phospholipase C-γ, but whether this phosphorylation event leads to a direct alteration of enzyme activity has not yet been demonstrated. Whether EGF receptors and G-protein-linked hormone receptors activate the same, different or overlapping phospholipase C isoenzymes also remains an important unresolved question. The present findings suggest that EGF receptors and non-tyrosine-kinase hormone receptors each possess the capacity to stimulate phospholipase(s) C activity in WB cells, but this apparently occurs by separate pathways.

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