Epidermal-growth-factor-induced formation of inositol phosphates in human A431 cells

Differences from the effect of bradykinin

Ben C. TILLY,*† Peter A. VAN PARIDON,† Ingrid VERLAAN,*§ Siegfried W. DE LAAT* and Wouter H. MOOLENAAR*§

*Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands, and †Department of Biochemistry, University of Utrecht, 3584 CH Utrecht, The Netherlands

In human A431 epidermoid carcinoma cells, epidermal growth factor (EGF) rapidly stimulates the breakdown of inositol phospholipids and raises cytoplasmic free $[Ca^{2+}]$. In this paper, we investigate the action of EGF on inositol phosphate metabolism, and we compare it with the previously described effects of bradykinin on the same cell system [Tilly, van Paridon, Verlaan, Wirtz, de Laat & Moolenaar (1987) *Biochem. J.* **244**, 129–135]. In cells prelabelled with $[^3H]$ inositol, EGF slowly but persistently (for at least 30 min) stimulates the formation of $[^3H]$ inositol phosphates, whereas bradykinin causes an immediate but transient release of inositol phosphates, which lasts for only a few minutes. The EGF effect is additive to bradykinin stimulation and does not require extracellular Ca^{2+} . In contrast, inositol phosphate formation induced by Ca^{2+} -ionophore A23187 has an absolute requirement for external Ca^{2+} . Treatment of the cells with 12-*O*-tetradecanoylphorbol 13-acetate completely abolishes the response to EGF and to sub-optimal doses of bradykinin, suggesting a negative-feedback function of protein kinase C. Pretreatment of the cells with pertussis toxin has no effect on inositol phosphate formation induced by either EGF or bradykinin. Unlike bradykinin, EGF stimulates very little accumulation of inositol 1,4,5-trisphosphate, with only a small and rather variable release of Ca^{2+} from intracellular stores. EGF rapidly but transiently increases inositol 1,3,4-trisphosphate and 1,3,4,5-tetrakisphosphate, but the effects are much smaller than those of bradykinin. In addition, EGF increases both inositol mono- and bis-phosphate. At 10 min after EGF addition, inositol monophosphate, unlike the other inositol phosphates, is still increasing. It is concluded that the EGF-dependent pattern of stimulation is different from that observed in bradykinin-stimulated A431 cells, suggesting that there are separate mechanisms of inositol-lipid hydrolysis involved.

INTRODUCTION

The binding of EGF to its plasma-membrane receptor initiates a variety of cellular responses, usually leading to enhanced DNA synthesis and cell division, but induction of biological effects unrelated to growth stimulation has also been demonstrated [1,2]. Although much has been learned about the molecular structure of the EGF receptor, relatively little is known about the nature of the intracellular signals that mediate the diverse biological responses to EGF. Among the immediate consequences of EGF binding are activation of the receptor tyrosine kinase [3], increases in cytoplasmic pH_i and free $[Ca^{2+}]$ [4–6] and enhanced turnover of inositol phospholipids [7–9]. The last response is particularly significant in human A431 carcinoma cells, which express a very high number of EGF receptors on their surface, but is usually undetectable in fibroblasts [10,11].

Previously we analysed the rapid formation of a variety of inositol phosphates in A431 cells stimulated

with the mitogenic nonapeptide bradykinin [12]. We found an immediate massive increase in $Ins(1,4,5)P_3$, together with a rapid release of Ca^{2+} from internal stores. In addition, bradykinin stimulation of A431 cells leads to formation of various other inositol phosphates, including $Ins(1,3,4,5)P_4$ and $Ins(1,3,4)P_3$.

We now have investigated the production of inositol phosphates in EGF-stimulated A431 cells, for which we have previously reported the occurrence of a rapid rise in $[Ca^{2+}]_i$ [6]. Our results indicate that the inositol phosphate pattern induced by EGF differs greatly from that evoked by bradykinin in the same cell system. The results suggest that EGF and bradykinin use separate mechanisms to evoke phosphoinositide hydrolysis.

MATERIALS AND METHODS

Reagents and radiochemicals

EGF (receptor grade) was purchased from Collaborative Research (Lexington, MA, U.S.A.) and pertussis

Abbreviations used: EGF, epidermal growth factor; $Ins(1)P$, inositol 1-phosphate; $Ins(1,4)P_2$, inositol 1,4-bisphosphate; $Ins(1,3,4)P_3$, inositol 1,3,4-trisphosphate; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; $Ins(1,3,4,5)P_4$, inositol 1,3,4,5-tetrakisphosphate; $PtdIns$, phosphatidylinositol; $PtdIns(4)P$, phosphatidylinositol 4-phosphate; $PtdIns(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; $[Ca^{2+}]_i$, intracellular Ca^{2+} concn.

† To whom reprint requests should be addressed.

§ Present address: The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands.

toxin from List Laboratories (Campbell, CA, U.S.A.). Other agents were from the following sources: A32187 from Calbiochem-Behring; TPA from Sigma; fetal-calf serum from Flow Laboratories; indo-1 acetoxymethyl ester from Molecular Probes (Eugene, OR, U.S.A.).

myo-[2-³H]Inositol (12.3 Ci/mmol), *D-myo*[2-³H]-inositol 1-phosphate (1 Ci/mmol), *D-myo*[2-³H]-inositol 1,4-bisphosphate (1 Ci/mmol) and *D-myo*[2-³H]-inositol 1,4,5-trisphosphate (1 Ci/mmol) were purchased from Amersham Corp. ¹²⁵I-EGF (200 µCi/µg) was from DuPont NEN Research (Dreieich, Germany). [³H]-Inositol 1,3,4,5-tetrakisphosphate was kindly provided by Dr. R. F. Irvine (Cambridge, U.K.).

Cell culture

A431 cells were routinely grown in Dulbecco's modified Eagle's medium containing 7.5% (v/v) fetal-calf serum. The cells were plated on 35 mm-diam. dishes (Costar) and grown to near-confluency before being used in the experiments.

Determination of total inositol phosphates

Cells were prelabelled to near isotopic equilibrium with 4 µCi of [³H]inositol/ml for 24 h. At 2 h before stimulation, the cells were incubated in serum-free Dulbecco's medium containing 20 mM-Hepes (pH 7.4). The cells were stimulated with EGF in the presence of 10 mM-LiCl for various periods, and the incubations were terminated by replacing the medium by 1 ml of 10% (v/v) trichloroacetic acid. After 10 min cell extracts were collected, and trichloroacetic acid was removed by washing with diethyl ether [12]. After neutralization with Tris base, the extracts were processed for analysis of the total [³H]inositol phosphate fraction (InsP_N fraction by anion-exchange chromatography on AG 1 × 8 columns (200–400 mesh, formate form; Bio-Rad) eluted with 1.0 M-ammonium formate/0.1 M-formic acid as previously described [12].

Separation of inositol phosphates by h.p.l.c.

The methods used to separate the various inositol phosphates were essentially similar to those described previously [12]. In brief, cells were prelabelled with 15 µCi of [³H]inositol for 24 h and inositol phosphates were separated on a Nucleosil 10 NH₂ h.p.l.c. column (4 mm × 250 mm; Macherey-Nagel). The column was eluted with a linear gradient to 1.5 M-ammonium formate/H₃PO₄ (pH 3.7), and ³H radioactivity was determined by liquid-scintillation counting. The column was calibrated with [³H]inositol phosphates [Ins(1)P, Ins(1,4)P₂, Ins(1,4,5)P₃ and Ins(1,3,4,5)P₄]. For further details see ref. [12].

¹²⁵I-EGF binding

Cells were incubated with ¹²⁵I-EGF (≈ 1 ng/ml) in Dulbecco's medium containing 0.1% (w/v) bovine serum albumin at 37 °C, as previously described [13]. Specific binding was determined over various incubation periods before cells were solubilized with 0.5 M-NaOH and ¹²⁵I radioactivity in the extracts was determined by scintillation counting.

Measurements of [Ca²⁺]_i

Nearly confluent A431 monolayers, attached to rectangular glass coverslips were loaded with indo-1 [14] by exposing them to 2 µM-indo-1 ester for 40 min at 37 °C,

as previously described for quin-2 loading [6]. [Ca²⁺]_i-dependent fluorescence was recorded and calibrated as described [6] at an excitation wavelength of 355 nm (5 nm slit) and an emission wavelength of 405 nm (10 nm slit) (see ref. [14]).

RESULTS

Effect of EGF on inositol phosphate formation in A431 cells: comparison with the effects of bradykinin and histamine

We first examined the production of inositol phosphates in EGF-stimulated A431 cells in the presence of 10 mM-LiCl. To determine the EGF dose-dependence, cell cultures were stimulated with various amounts of EGF for 30 min and inositol phosphate accumulation was quantified. Half-maximal stimulation was observed at an EGF concentration of ≈ 15 ng/ml (2.5 nM), and the response became saturated at 50–100 ng/ml. At this concentration the inositol phosphate concentration is about 3 times the basal value (Fig. 1). The EGF concentration-dependence for the inositol phosphate response is very similar to that for other reported effects on A431 cells, such as Ca²⁺ signalling [6], stimulation of Na⁺/H⁺ exchange [4] and tyrosine-specific protein phosphorylation [15].

The time course of formation of inositol phosphates in response to EGF is illustrated in Fig. 2. There is an apparent lag period of 2–5 min after addition of a saturating dose of EGF (100 ng/ml) before accumulation of inositol phosphates becomes detectable. Thereafter, a slow but prolonged increase in inositol phosphates is observed, which continues for at least 30 min after EGF

Fig. 1. Dose-dependency of EGF-induced inositol phosphate accumulation

myo-[³H]Inositol-prelabelled A431 cells were stimulated with the indicated concentrations of EGF for 30 min in the presence of 10 mM-LiCl. The incubations were terminated and an inositol phosphate fraction (InsP_N fraction, containing InsP, InsP₂ and InsP₃) was prepared as previously described [12]. Data are expressed as means ± S.E.M. (n = 3).

Fig. 2. Time course of EGF-stimulated inositol phosphate production

myo-[^3H]inositol prelabelled cells were incubated in 10 mM-LiCl medium for 30 min. Cells were incubated with EGF (100 ng/ml; ●) or bradykinin [BK; 1 μM (○) or 20 nM (▽)] for the indicated times. The InsP_N fraction was prepared as described elsewhere [12]. Each data point represents the mean $\pm$ S.E.M. ($n = 3$).

Fig. 3. Additive effects of EGF, bradykinin and histamine on inositol phosphate formation

EGF, bradykinin (BK) or histamine (HIST) (100 ng/ml, 2 μM and 100 μM respectively) were added to cells pre-stimulated with bradykinin (1 μM) for 5 min. All cultures were incubated in a 10 mM-LiCl medium for 30 min. Arrow indicates the 5 min time point of hormone addition. Results are expressed as means $\pm$ S.E.M. ($n = 3$).

addition. The initial lag period could not be decreased by increasing the EGF concentration to 250 ng/ml (results not shown).

We compared the time course of the response to EGF with that to bradykinin, a potent inducer of polyphosphoinositide hydrolysis in A431 cells [12]. As shown in Fig. 2, there is a striking difference between the effects of EGF and bradykinin on the kinetics of inositol

Fig. 4. H.p.l.c. analysis of EGF-induced inositol phosphate formation

myo-[^3H]inositol-prelabelled cultures were incubated for the indicated times with EGF (100 ng/ml) in the absence of Li^+ . Analysis of the individual inositol phosphates by anion-exchange h.p.l.c. was done as previously described [12]. All data points are from one representative experiment ($n = 7$).

phosphate formation. The accumulation of inositol phosphates caused by bradykinin is detectable as early as 7 s after hormone addition and is terminated in less than 2 min, suggesting that the phospholipase C pathway is desensitized to bradykinin very rapidly [12]. This apparent desensitization is not attributable to depletion of the hormone-sensitive phosphoinositide pool, since it also occurs after addition of sub-maximal doses of bradykinin (Fig. 2). In contrast, the response to EGF develops more slowly, without any sign of desensitization over a time course of 30 min. From these kinetic data we conclude that, whereas bradykinin stimulates the release of inositol phosphates only transiently (< 2 min), EGF evokes persistent (> 30 min) hydrolysis of inositol phospholipids.

To determine whether the effects of EGF and bradykinin are additive, we added EGF to cells pre-stimulated with 1 μM -bradykinin for 5 min. Fig. 3 shows that, in cells exposed to bradykinin, EGF is still capable of evoking an additional prolonged increase in inositol phosphates, starting, again, after a 2 min latency. As expected, a second application of bradykinin has no effect (Fig. 3). In other words, the responses to saturating

doses of EGF and bradykinin are additive. Histamine, a hormone that resembles bradykinin in evoking PtdInsP_2 breakdown and raising $[\text{Ca}^{2+}]_i$ in A431 cells ([9]; W. H. Moolenaar, unpublished work), also evokes a further accumulation of inositol phosphates when added 5 min after bradykinin (Fig. 3). Like the response to bradykinin, histamine-induced inositol phosphate formation occurs without a detectable lag period. In addition, a second, more prolonged, phase of inositol phosphate formation is observed, starting approx. 5 min after histamine addition. The simplest explanation of these results is that each individual hormone receptor (EGF, bradykinin, histamine) uses its own separate pool of phosphoinositides to generate inositol phosphates.

Kinetic studies of inositol phosphates separated by h.p.l.c.

We have separated the individual $[^3\text{H}]$ inositol phosphates and analysed the kinetics of their formation and degradation in response to EGF by means of h.p.l.c. anion-exchange chromatography. In a previous study [12], we identified various inositol phosphate peaks in A431 cells, including InsP , InsP_2 , two InsP_3 isomers (1,3,4 and 1,4,5) and InsP_4 . Fig. 4 shows how the individual inositol phosphate amounts vary with time between 0 and 10 min after addition of EGF (100 ng/ml) in the absence of any Li^+ . In the experiment illustrated, there is no significant increase in $\text{Ins}(1,4,5)\text{P}_3$, as was the case in four other experiments. In two experiments, however, we did observe a small but significant increase in $\text{Ins}(1,4,5)\text{P}_3$ (52% and 55%, respectively) within 1 min of EGF addition. However, this EGF-dependent accumulation of $\text{Ins}(1,4,5)\text{P}_3$ is very small when compared with the bradykinin-induced $\text{Ins}(1,4,5)\text{P}_3$ formation (approx. 10 times basal values; cf. [12]). These findings are consistent with the rather variable contribution of intracellularly stored Ca^{2+} to the EGF-induced $[\text{Ca}^{2+}]_i$ rise as measured with indo-1 fluorescence (see the next section). In contrast, a transient accumulation of both $\text{Ins}(1,3,4)\text{P}_3$ and InsP_4 was readily observed in all experiments (Fig. 4). In Fig. 5 we compare the time

Fig. 5. EGF-induced formation of InsP_3 .

Accumulation of $\text{Ins}(1,3,4)\text{P}_3$ (○) and $\text{Ins}(1,4,5)\text{P}_3$ (●) was monitored during the first 30 s after EGF (100 ng/ml) addition.

courses of the formation of both InsP_3 isomers in more detail; the EGF-induced increase in $\text{Ins}(1,3,4)\text{P}_3$ starts only after a distinct lag period of approx. 7–15 s (Fig. 5), whereas an increase in the 1,4,5 isomer could barely be measured, even during the first seconds of receptor stimulation. The basal amounts of both $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ varied considerably among different batches of A431 cells; these variations affect the EGF effect on $\text{Ins}(1,3,4)\text{P}_3$ only in a quantitative rather than in a qualitative way.

Almost immediately after EGF addition, there is a more than 2-fold increase in InsP_2 (Fig. 4). Maximal values are reached within 30 s and are maintained for at least 10 min. In addition, Fig. 4 shows that EGF induces a slow but continued formation of InsP in A431 cells. Even 10 min after EGF addition InsP is still increasing (Fig. 4), whereas the other inositol phosphates are no longer accumulating. These results, taken together with those from the studies of Li^+ -treated cells (Fig. 2), suggest that the prolonged increase in InsP in A431 cells during EGF stimulation is caused by a small but persistent hydrolysis of phosphoinositides (PtdIns , PtdInsP_2), rather than by transient breakdown of PtdInsP_2 .

EGF-induced rise in $[\text{Ca}^{2+}]_i$

We have reported previously that EGF rapidly but transiently raises $[\text{Ca}^{2+}]_i$ in A431 cells as measured with the fluorescent Ca^{2+} indicator quin-2 [6]. In Ca^{2+} -free media, this EGF effect was almost completely abolished, whereas the $[\text{Ca}^{2+}]_i$ rise induced by serum [6] was hardly decreased. These results led us to conclude that EGF stimulates Ca^{2+} influx rather than Ca^{2+} release from internal stores [6]. We have now re-investigated the nature of the EGF-induced Ca^{2+} signal in A431 cells, using the novel dye indo-1, which can be used at much lower intracellular concentrations than quin-2 [14]. As shown in Fig. 6, removal of extracellular Ca^{2+} by EGTA inhibits largely but not completely the EGF-induced Ca^{2+} signal in indo-1-loaded cells, indicating a contribution from intracellularly stored Ca^{2+} , whereas the bradykinin-evoked signal is hardly attenuated (Fig. 6). It should be emphasized, however, that the magnitude of the EGF-dependent $[\text{Ca}^{2+}]_i$ rise measured in both normal and Ca^{2+} -free media was highly variable between different experiments, with some cell cultures responding as in Fig. 6 and other cultures showing hardly any apparent release of stored Ca^{2+} (results not shown). Such variability was never observed with bradykinin or serum as stimuli [6, 13]. Furthermore, we note that the peak value of the EGF-induced Ca^{2+} signal was substantially smaller than that of the bradykinin response.

Effect of extracellular Ca^{2+} on inositol phosphate formation by EGF and A23187

In initial experiments on A431 cells, Sawyer & Cohen [7] reported that (i) external Ca^{2+} is required for the stimulation of PtdIns turnover by EGF, and (ii) Ca^{2+} influx (via ionophore A23187) is sufficient for the PtdIns response. We therefore examined the requirement of extracellular Ca^{2+} for inositol phosphate production induced by both EGF and A23187. As illustrated in Table 1, removal of Ca^{2+} from the incubation medium by using excess EGTA has no significant effect on the accumulation of inositol phosphates evoked by EGF. Table 1 also illustrates that addition of A23187 (0.5 μM)

Fig. 6. Comparison of the EGF-induced rise in $[Ca^{2+}]_i$ with the effect of bradykinin

Indo-1-loaded cells were stimulated with external EGF (100 ng/ml) and bradykinin (BK; 1 μ M) in the presence or absence of Ca^{2+} . Ca^{2+} was removed by addition of 4 mM-EGTA before EGF addition.

Table 1. Effects of extracellular Ca^{2+} on inositol phosphate formation by EGF and A23187

Cells were stimulated with EGF or A23187 for 10 min in the presence of 10 mM-LiCl. Extracellular Ca^{2+} was removed by adding 4 mM-EGTA. Data are expressed as means $\pm$ S.E.M. ($n = 3$).

Addition	$[^3H]$ Inositol phosphates (d.p.m.)	
	Normal medium	Ca^{2+} -free medium
None	6061 $\pm$ 308	7054 $\pm$ 429
EGF (100 ng/ml)	13389 $\pm$ 263	12756 $\pm$ 516
A23187 (0.5 μ M)	12925 $\pm$ 541	7383 $\pm$ 281

to A431 cells, which leads to a massive and sustained increase in $[Ca^{2+}]_i$ [6], results in enhanced accumulation of inositol phosphates to approximately the same extent as with EGF. In contrast with the results obtained with EGF, removal of external Ca^{2+} completely blocks the effect of A23187 on inositol phosphate production (Table 1). Under such Ca^{2+} -free conditions, A23187 has been shown to evoke a transient rather than a sustained $[Ca^{2+}]_i$ rise in A431 cells, owing to the release of internal Ca^{2+} stores [6]. When taken together, our results indicate that a prolonged increase in $[Ca^{2+}]_i$ can trigger phosphoinositide hydrolysis, whereas a transient Ca^{2+} signal cannot. The nature of the individual inositol phosphates released by ionophore A23187 remains to be investigated.

Effects of TPA

In many cell systems, phorbol esters and synthetic diacylglycerols, acting via protein kinase C, inhibit

Table 2. Effects of TPA pre-treatment on inositol phosphate production

$[^3H]$ Inositol-pre-labelled cells were treated with TPA (50 ng/ml; 30 min) before stimulation with either EGF or bradykinin. Results are expressed as percentages of control; control represents 1606 $\pm$ 40 d.p.m. (mean $\pm$ S.E.M.; $n = 3$).

Addition	$[^3H]$ Inositol phosphates (% of control)	
	Control	+ TPA
None	100 $\pm$ 3	98 $\pm$ 7
EGF (100 ng/ml)	200 $\pm$ 5	101 $\pm$ 4
Bradykinin (20 nM)	190 $\pm$ 7	111 $\pm$ 9
Bradykinin (1 μ M)	263 $\pm$ 4	191 $\pm$ 4

phosphoinositide hydrolysis induced by extracellular stimuli [16–18]. In A431 cells, TPA has been shown to inhibit the EGF-induced $[Ca^{2+}]_i$ rise [6] and Na^+/H^+ -exchange activity [19] and to attenuate the EGF-receptor tyrosine kinase activity [3,20]. Also, in hepatocytes pretreatment with TPA blocks the Ca^{2+} response to EGF [21]. Table 2 shows that pretreatment of A431 cells with TPA (50 ng/ml for 30 min) completely blocks the inositol phosphate response to EGF, whereas basal inositol phosphate contents remain unchanged. Since TPA pretreatment can affect the binding of EGF to its receptor [22], we measured the rate of specific ^{125}I -EGF binding to A431 cells during the first 60 min at 37 $^{\circ}C$, and found that the initial rate of EGF binding is only slightly decreased (less than 10%) in the presence of TPA (results not shown).

Unlike the response to saturating doses of EGF, the inositol phosphate accumulation induced by a saturating concentration of bradykinin (1 μ M) is decreased by only 25–30% in TPA-treated A431 cells. However, when the bradykinin concentration is lowered to 20 nM, which gives a similar quantitative response to EGF (Fig. 2), TPA completely inhibits the inositol-lipid breakdown (Table 2).

Effect of pertussis toxin

In a few cell types, there is evidence for the involvement of a pertussis-toxin-sensitive GTP-binding protein in receptor-mediated activation of phospholipase C [23,24]. In many other systems, however, including bradykinin-stimulated A431 cells [12], this bacterial toxin is ineffective in attenuating receptor-linked phosphoinositide breakdown. We found that preincubation of A431 cells with pertussis toxin (200 ng/ml) for 2 h did not affect either the basal or the EGF-induced formation of inositol phosphates. A more prolonged treatment ($\sim$ 20 h) with pertussis toxin was found to lower basal amounts by less than $\sim$ 20%, without changing the relative stimulation induced by EGF (results not shown).

DISCUSSION

Several studies have provided evidence for the involvement of inositol phospholipid breakdown in growth-factor

action [7,25–28]. This concept is well documented for mitogens such as platelet-derived growth factor [26], thrombin [27] and bombesin [28], but the ability of EGF to stimulate inositol-lipid breakdown is rather cell-type specific. In fibroblasts, for example, EGF stimulates the synthesis of PtdInsP and PtdInsP₂ [29], without detectably increasing inositol phosphates [11,30]. In epidermoid A431 cells, on the other hand, EGF not only enhances the rate of [³²P]P_i incorporation into PtdIns [7], but also stimulates diacylglycerol formation [31] and protein kinase C activity [32]. More recently, Pike & Eakes [8] showed that EGF stimulates PtdInsP₂ breakdown as well as formation of PtdInsP, and Hepler *et al.* [9] reported an accumulation of inositol phosphates accompanied by a rise in [Ca²⁺]_i. In the present paper, we have analysed the action of EGF on inositol phosphate metabolism in A431 cells and we have compared it with our previous results on the mode of action of bradykinin on the same cell system [12]. Our present findings clearly show that EGF stimulates inositol phosphate formation in A431 cells, consistent, at least qualitatively, with the findings of Pike & Eakes [8] and Hepler *et al.* [9], although there are also significant discrepancies with these previous studies, not only with respect to the kinetics and magnitude of the PtdIns response with bradykinin versus EGF, but also with respect to the accumulation of Ins(1,4,5)P₃.

Our analysis of the time course of EGF-induced phosphoinositide breakdown by preparing a total inositol phosphate fraction (InsP_N fraction, containing mainly InsP) shows a distinct lag of a few minutes between EGF addition and InsP_N accumulation (Fig. 2). Thereafter, EGF-induced InsP_N formation proceeds in a linear fashion for at least 30 min. This time course of InsP_N accumulation is very different from that evoked by bradykinin, which shows no detectable lag period and reaches a plateau ('homologous desensitization') as early as 2 min after hormone addition. These results imply that, whereas bradykinin stimulates phospholipase C activity in a transient way (< 2 min), EGF-induced phosphoinositide hydrolysis has a very low rate at early time points (0–4 min) and then proceeds at an accelerated and steady rate.

From our h.p.l.c. and indo-1 data it appears that, during the 1 min after EGF addition, there is already some formation of InsP₃ and InsP₄ as well as a release of Ca²⁺ from intracellular stores. In most (5/7) of our experiments, however, maximal concentrations of EGF failed to stimulate a significant accumulation of Ins(1,4,5)P₃. By contrast, a transient accumulation of Ins(1,3,4)P₃ and InsP₄ was always detectable in EGF-treated cells. We note that formation of these inositol phosphates occurs rapidly, without a detectable lag period, whereas a lag is prominent when the total InsP_N fraction is measured (in the presence of Li⁺). Most likely the elevated basal amounts of InsP_N, caused by Li⁺ pre-treatment, mask the initial increase in InsP₃ and InsP₄. A similar time course of InsP accumulation in the presence of Li⁺ was observed by Wahl *et al.* [33], but not by Hepler *et al.* [9].

The lack of a substantial increase in Ins(1,4,5)P₃ agrees with our results obtained with the Ca²⁺ indicator indo-1 (Fig. 6), showing a rather small and highly variable contribution of intracellularly stored Ca²⁺ to the EGF-induced [Ca²⁺]_i rise. (When quin-2 is used as a Ca²⁺ indicator, this component of the Ca²⁺ signal is even

undetectable [6].) Furthermore, we concur with others who emphasized that measurements of total cellular concentrations of Ins(1,4,5)P₃ may not reflect the biologically relevant concentrations close to intracellular Ca²⁺ stores just underneath the plasma membranes [34]. We therefore conclude that EGF is a rather weak inducer of PtdInsP₂ hydrolysis in A431 cells, giving rise to a relatively small, localized, amount of Ins(1,4,5)P₃, which then is rapidly metabolized to both InsP₂ and InsP₄/Ins(1,3,4)P₃ via pathways very similar to those described for various other hormone-sensitive cell systems [35–38]. In the experiments reported by Hepler *et al.* [9], EGF was more potent in generating Ins(1,4,5)P₃ and releasing Ca²⁺ from internal stores than in the present study, whereas the effects of bradykinin are more or less similar. Most probably there are multiple clones of A431 cells that respond differently to EGF.

Although the biochemical pathways underlying the formation of InsP₃ and InsP₄ by either agonist are likely to be qualitatively similar, important differences are observed in the patterns of InsP and InsP₂ formation after stimulation with EGF as compared with bradykinin. We found that the EGF-induced increases in InsP and InsP₂ are sustained rather than transient, as observed with bradykinin as a stimulus (cf. ref. [12]). These persistently increased amounts do not simply reflect a new steady-state condition, since the accumulation of InsP_N (in the presence of Li⁺) continues for at least 30 min. One possible explanation is that, in addition to PtdInsP₂ breakdown, EGF evokes a direct and persistent hydrolysis of PtdIns and/or PtdInsP, as suggested for other cell systems [38–40]. However, we cannot exclude the possibility that the observed changes in inositol phosphates are due to the continued breakdown of PtdInsP₂ at a very slow rate.

Our results argue against a role of the EGF-induced [Ca²⁺]_i rise in triggering the formation of inositol phosphates as described by others [7,33]. First, the EGF-induced accumulation of inositol phosphates is independent of extracellular Ca²⁺. Second, when intracellularly stored Ca²⁺ is released by using ionophore A23187 in Ca²⁺-free media, there is no significant hydrolysis of inositol lipids. Hence, although PtdIns turnover may be Ca²⁺-dependent [7] and a sustained increase in [Ca²⁺]_i is capable of activating phospholipase C (Table 1), the formation of inositol phosphates by EGF is not secondary to a rise in [Ca²⁺]_i.

The observed inhibitory action of TPA on inositol phosphate formation induced by EGF and at non-saturating concentrations of bradykinin seems to reflect a rather universal negative-feedback function of protein kinase C, the cellular receptor for TPA, in uncoupling receptors from second-messenger-generating systems. Whether protein kinase C exerts its inhibitory effect at the level of phospholipase C and/or a putative G-protein or at the level of the hormone receptor (see, e.g., [41,42]), or both, remains to be determined.

Finally, it is noteworthy and perhaps somewhat unexpected that a potent stimulus of the phospholipase C/protein kinase C pathway, such as bradykinin, expected to activate protein kinase C, fails to mimic the effect of the kinase C activator TPA on EGF-receptor 'inactivation'. Perhaps sustained activation of kinase C (by TPA) rather than a more transient activation (by endogenously produced diacylglycerol) might explain these different results.

We thank Dr. K. W. A. Wirtz for helpful discussions and Dr. R. F. Irvine for providing [^3H]InsP $_4$. This work was supported in part by the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds).

REFERENCES

1. Carpenter, G. & Cohen, S. (1979) *Annu. Rev. Biochem.* **48**, 193–216
2. Carpenter, G. (1984) *Cell* **37**, 357–358
3. Hunter, T. & Cooper, J. A. (1985) *Annu. Rev. Biochem.* **54**, 897–930
4. Rothenberg, P., Glaser, L., Schlessinger, P. & Cassel, D. (1983) *J. Biol. Chem.* **258**, 12644–12653
5. Hesketh, T. R., Moore, J. P., Morris, J. D. H., Taylor, M. V., Rogers, J., Smith, G. A. & Metcalfe, J. C. (1985) *Nature (London)* **313**, 481–484
6. Moolenaar, W. H., Aerts, R. J., Tertoolen, L. G. J. & de Laat, S. W. (1986) *J. Biol. Chem.* **261**, 279–284
7. Sawyer, S. T. & Cohen, S. (1981) *Biochemistry* **20**, 6280–6286
8. Pike, L. J. & Eakes, A. T. (1987) *J. Biol. Chem.* **262**, 1644–1651
9. Hepler, J. R., Nakahato, N., Lovenberg, T. W., Di Guiseppe, J., Herman, B., Earp, H. S. & Harden, T. K. (1987) *J. Biol. Chem.* **262**, 2951–2956
10. Macphee, C., Drummond, A., Otto, A. & Jimenez de Asua, L. (1984) *J. Cell. Physiol.* **119**, 35–40
11. Besterman, J. M., Watson, S. P. & Cuatrecasas, P. (1986) *J. Biol. Chem.* **261**, 723–727
12. Tilly, B. C., van Paridon, P. A., Verlaan, I., Wirtz, K. W. A., de Laat, S. W. & Moolenaar, W. H. (1987) *Biochem. J.* **244**, 129–135
13. Moolenaar, W. H., Yarden, Y., de Laat, S. W. & Schlessinger, J. (1982) *J. Biol. Chem.* **257**, 8502–8506
14. Gryniewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
15. Hunter, T. & Cooper, J. A. (1981) *Cell* **24**, 741–752
16. Brock, T. A., Rittenhouse, S. E., Powers, C. W., Ekstein, L. S., Gimbrone, M. A. & Alexander, R. W. (1985) *J. Biol. Chem.* **260**, 14158–14162
17. Orellana, S. A., Solski, P. A. & Brown, J. H. (1985) *J. Biol. Chem.* **260**, 5236–5239
18. Leeb-Lundberg, L. M. F., Cotecchia, S., Lomasney, J. W., DeBernardis, J. F., Lefkowitz, R. J. & Caron, M. G. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 5651–5655
19. Whiteley, B., Cassel, D., Zhuang, Y. & Glaser, L. (1984) *J. Cell Biol.* **99**, 1162–1166
20. Cochet, C., Gill, G. N., Meisenhelder, J., Cooper, J. A. & Hunter, T. (1984) *J. Biol. Chem.* **259**, 2553–2558
21. Johnson, R. M., Cannelly, P. A., Sisk, R. B., Pobiner, B. F., Hewlett, E. L. & Garrison, J. C. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 2032–2036
22. Davis, R. J. & Czech, M. P. (1984) *J. Biol. Chem.* **259**, 8545–8549
23. Nakamura, T. & Ui, M. (1985) *J. Biol. Chem.* **260**, 3584–3593
24. Volpi, M., Naccache, P. H., Molski, T. F. P., Shefcyk, J., Huang, C., Marsh, M. L., Munoz, J., Becker, E. L. & Sha'afi, R. I. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2708–2712
25. Berridge, M. J. & Irvine, R. F. (1984) *Nature (London)* **312**, 315–321
26. Berridge, M. J., Heslop, J. P., Irvine, R. F. & Brown, K. D. (1984) *Biochem. J.* **222**, 195–201
27. Paris, S. & Pouyssegur, J. (1986) *EMBO J.* **5**, 55–60
28. Takuwa, N., Takuwa, Y., Bollag, W. E. & Rasmussen, H. (1987) *J. Biol. Chem.* **262**, 182–188
29. Raben, D. M. & Cunningham, D. D. (1985) *J. Cell. Physiol.* **125**, 582–590
30. L'Allemain, G. & Pouyssegur, J. (1986) *FEBS Lett.* **197**, 344–348
31. Smith, K. B., Losenczy, I., Sahai, A., Pannerselvam, M., Fehnel, P. & Salomon, D. S. (1983) *J. Cell. Physiol.* **117**, 91–100
32. Whiteley, B. & Glaser, L. (1986) *J. Cell Biol.* **103**, 1355–1362
33. Wahl, M. I., Sweatt, J. D. & Carpenter, G. (1987) *Biochem. Biophys. Res. Commun.* **142**, 688–695
34. Merritt, J. E., Taylor, C. W., Rubin, R. P. & Putney, J. W., Jr. (1986) *Biochem. J.* **238**, 825–829
35. Heslop, J. P., Irvine, R. F., Tashjian, A. T. & Berridge, M. J. (1985) *J. Exp. Biol.* **119**, 395–402
36. Batty, I. R., Nahorski, S. R. & Irvine, R. F. (1985) *Biochem. J.* **232**, 211–215
37. Irvine, R. F., Letcher, A. J., Heslop, J. P. & Berridge, M. J. (1986) *Nature (London)* **320**, 631–634
38. Hansen, G. A., Mah, S. & Williamson, J. R. (1986) *J. Biol. Chem.* **261**, 8100–8103
39. Wilson, D. B., Neufeld, E. J. & Majerus, P. W. (1985) *J. Biol. Chem.* **260**, 1046–1051
40. Imai, A. & Gershengorn, M. C. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8540–8544
41. Friedman, B., Frackelton, A. R., Ross, A. M., Connors, J. M., Fujiki, H., Sugimura, T. & Rosner, M. R. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3034–3038
42. Hunter, T., Ling, N. & Cooper, J. A. (1984) *Nature (London)* **311**, 480–483

Received 7 October 1987/15 February 1988; accepted 4 March 1988