Inositol phosphate metabolism in bradykinin-stimulated human A431 carcinoma cells

Relationship to calcium signalling

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Stimulation of human A431 epidermoid carcinoma cells by bradykinin causes a very rapid release of inositol phosphates and a transient rise in cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Bradykinin-induced inositol phosphate formation is half-maximal at a concentration of 4 nM and is not affected by pertussis toxin. H.p.l.c. analysis of the various inositol phosphates shows an immediate but transient accumulation of inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$], which reaches a peak value of approx. 10 times the basal level within 15 s and slightly precedes the rise in $[\text{Ca}^{2+}]_i$, both parameters changing in parallel. After a lag period, bradykinin also induces a massive accumulation of $\text{Ins}(1,3,4)\text{P}_3$ and inositol 1,3,4,5-tetrakisphosphate [$\text{Ins}(1,3,4,5)\text{P}_4$]. Our data support the view that part of the newly formed $\text{Ins}(1,4,5)\text{P}_3$ is converted into $\text{Ins}(1,3,4)\text{P}_3$ by phosphorylation/dephosphorylation with $\text{Ins}(1,3,4,5)\text{P}_4$ as intermediate. Furthermore, A431 cells were found to contain strikingly high basal levels of two other inositol phosphates, presumably inositol pentakisphosphate (InsP_5) and inositol hexakisphosphate (InsP_6), representing more than 50% of the total ^3H radioactivity incorporated into inositol phosphates. The presumptive InsP_5 and InsP_6 are only slightly affected by bradykinin. Although $\text{Ins}(1,3,4)\text{P}_3$ and InsP_4 could function as second messengers, our results suggest that, unlike $\text{Ins}(1,4,5)\text{P}_3$, neither $\text{Ins}(1,3,4)\text{P}_3$ nor InsP_4 are involved in Ca^{2+} mobilization.

INTRODUCTION

Receptor-mediated hydrolysis of phosphoinositides is a common cellular response to a variety of extracellular stimuli such as hormones, neurotransmitters and certain growth factors (Michell *et al.*, 1981; Berridge, 1984). There is now general agreement that receptor stimulation induces the phosphodiesteratic cleavage of $\text{PtdIns}(4,5)\text{P}_2$, resulting in the formation of at least two second messengers: diacylglycerol, an activator of protein kinase C (Nishizuka, 1983), and $\text{Ins}(1,4,5)\text{P}_3$, which triggers the release of Ca^{2+} from intracellular stores (Berridge & Irvine, 1984). It has recently become clear that in addition to $\text{Ins}(1,4,5)\text{P}_3$, stimulated cells also produce $\text{Ins}(1,3,4)\text{P}_3$ (Irvine *et al.*, 1984, 1985; Burgess *et al.*, 1985) and $\text{Ins}(1,3,4,5)\text{P}_4$ (Batty *et al.*, 1985; Heslop *et al.*, 1985) via a pathway that apparently involves a specific $\text{Ins}(1,4,5)\text{P}_3$ 3-kinase (Irvine *et al.*, 1986), but whether these novel inositol phosphates have a second messenger role in Ca^{2+} signalling remains to be established.

In a recent study we have investigated the Ca^{2+} -mobilizing mechanisms of epidermal growth factor (EGF) in human A431 epidermoid carcinoma cells (Moolenaar *et al.*, 1986) for which EGF-induced phosphoinositide turnover had previously been described (Sawyer & Cohen, 1981). In a further series of

experiments we found that the nonapeptide bradykinin (Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg) not only is much more potent than EGF in raising $[\text{Ca}^{2+}]_i$ and inositol phosphate levels, but, unlike EGF, also stimulates DNA synthesis and cell division in A431 cells (W. H. Moolenaar & B. C. Tilly, unpublished work). Hence, bradykinin-activated A431 cells serve as a useful system to unravel the complex metabolism of inositol phospholipids and to examine their role in the control of cell proliferation.

In the present study we report the rapid formation of inositol phosphates in A431 cells stimulated with bradykinin. Complete separation of the individual inositol phosphates was obtained, allowing detailed analysis of their production and comparison with the time course of the Ca^{2+} signal.

MATERIALS AND METHODS

Radioisotopes

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 International.

Abbreviations used: EGF, epidermal growth factor; GroPIns , glycerophosphoinositol; $\text{Ins}(1)\text{P}$, inositol 1-phosphate; $\text{Ins}(1,4)\text{P}_2$, inositol 1,4-bisphosphate; $\text{Ins}(1,3,4)\text{P}_3$, inositol 1,3,4-trisphosphate; $\text{Ins}(1,4,5)\text{P}_3$, inositol 1,4,5-trisphosphate; $\text{Ins}(1,3,4,5)\text{P}_4$, inositol 1,3,4,5-tetrakisphosphate; InsP_5 , inositol pentakisphosphate; InsP_6 , inositol hexakisphosphate; PtdInsP_2 , phosphatidylinositol 4,5-bisphosphate; $[\text{Ca}^{2+}]_i$, cytoplasmic free Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle's medium.

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Chemicals

Bradykinin was obtained from Sigma, pertussis toxin from List Laboratories (Campbell, CA, U.S.A.) and quin-2 acetoxymethyl ester from Calbiochem. All chemicals used were analytical grade.

Cell culture

Human A431 epidermoid carcinoma cells (obtained from Dr. G. Todaro) were grown in DMEM (Gibco) supplemented with 7.5% (v/v) fetal calf serum. The cells were plated on 35 mm culture 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 μ Ci of [3 H]inositol/ml for 24 h. At 2 h prior to stimulation the cells were incubated in serum-free DMEM containing 20 mM-Hepes (pH 7.4). The cells were stimulated with bradykinin in the presence of 10 mM-LiCl for various periods and the incubations were terminated by replacing the medium by 1 ml of 10% trichloroacetic acid. After 10 min the extracts, containing the inositol phosphates, were collected and the trichloroacetic acid was removed by washing four times with 2 vol. of water-saturated diethyl ether. The samples were neutralized by adding Tris base, diluted to 4 ml with distilled water and placed on an AG 1X8 column (200–400 mesh, formate form; Bio-Rad) with a bed volume of 250 μ l. After eluting the column with 2 ml of water and 5 ml of 60 mM-ammonium formate/5 mM-sodium tetraborate, to remove inositol and glycerophosphoinositol, an inositol phosphate fraction was obtained by eluting five times with 2 ml of 1.0 M-ammonium formate/0.1 M-formic acid. This inositol phosphate fraction (Ins P_n fraction) consists of Ins P_1 , Ins P_2 and Ins P_3 (Berridge *et al.*, 1982).

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

To investigate inositol phosphate metabolism the cells were prelabelled with 15 μ Ci of *myo*-[2- 3 H]inositol/ml for 24 h. The cells were stimulated with bradykinin in the absence of LiCl and samples were prepared as described above. EGTA and mannitol was added to the neutralized samples to a final concentration of 1 mM and 50 μ M, respectively. Inositol phosphates were separated by h.p.l.c. anion-exchange chromatography according to a modification of the method described by Irvine *et al.* (1985), using a weakly basic anion exchange column (Nucleosil 10 NH $_2$; 4 mm $\times$ 250 mm; Macherey-Nagel). The column was eluted with a 45 ml linear gradient to 1.5 M-ammonium formate/phosphoric acid (pH 3.7), started after 10 ml of distilled water, at a flow rate of 1.25 ml/min. Adenine nucleotides (AMP, ADP and ATP) were added to the samples. The column was calibrated with 3 H-labelled inositol phosphates [Ins(1) P , Ins(1,4) P_2 and Ins(1,4,5) P_3 ; Amersham]. A_{254} was monitored. For determination of the inositol phosphates (except for Ins P_3), 625 μ l (0.5 min) fractions were taken. To quantify the 3 H radioactivity in Ins(1,3,4) P_3 and Ins(1,4,5) P_3 125 μ l (0.1 min) fractions were collected. 3 H radioactivity was determined by liquid-scintillation counting, using Instagel (Packard)/methanol/water (10:1:1, by vol.) as counting fluid (Heslop *et al.*, 1985).

Quin-2 fluorescence

Nearly confluent A431 monolayers, attached to rectangular glass coverslips, were loaded with quin-2 by incubating them in serum-free DMEM containing 10 μ M-quin-2 ester for 30 min at 37 $^{\circ}$ C. Fluorescence from the quin-2-loaded cells was continuously monitored in a Perkin-Elmer model 3000 spectrofluorometer, as previously described (Moolenaar *et al.*, 1986). For calibration procedures and other experimental details see Moolenaar *et al.* (1984, 1986).

RESULTS

Bradykinin-induced formation of inositol phosphates

Addition of bradykinin to nearly confluent cultures of A431 cells in the presence of 10 mM-LiCl causes a dose-dependent accumulation of inositol phosphates (Fig. 1). Half-maximal stimulation occurs at a concentration of 4 nM, which is close to the reported K_d for [3 H]bradykinin-receptor binding in various mammalian tissues (Innis *et al.*, 1981), while a saturating response is observed at approx. 1 μ M. At this concentration the inositol phosphate level is 3–4-fold that of the basal level. The stimulatory effect of bradykinin on inositol phosphate formation is very rapid (Fig. 2). Within 15 s of hormone stimulation, a 2–3-fold increase in inositol phosphates is measured. An apparent saturation of inositol phosphate accumulation occurs in less than 2 min. After that period no further production of inositol phosphates is detectable, despite the continuous presence of the hormone.

Calcium mobilization by bradykinin

Bradykinin stimulation of A431 cells immediately increases the [Ca^{2+}] $_i$, as measured by quin-2 fluorescence

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

myo-[3 H]inositol-prelabelled A431 cells were incubated with the indicated concentrations of bradykinin for 10 min in the presence of 10 mM-LiCl. The incubations were terminated and an inositol phosphate fraction (Ins P_n fraction, containing Ins P , Ins P_2 and Ins P_3) was prepared as described under 'Materials and methods'. Data are expressed as means $\pm$ S.E.M. for three determinations.

Fig. 2. Time course of bradykinin-mediated inositol phosphate formation

[^3H]Inositol-prelabelled cells were incubated in a medium containing 10 mM-LiCl for 30 min. Bradykinin (final concentration 1 μM) was added to the cultures at the indicated times prior to termination. An inositol phosphates-containing fraction was prepared as described under 'Materials and methods'. Each point represents the mean $\pm$ S.E.M. for triplicate cultures.

Fig. 3. Time course of bradykinin-induced Ca^{2+} signal

Quin-2-loaded cells were stimulated with 1 μM -bradykinin at time zero (arrow). Prior to quin-2 loading the cells were incubated in serum-free medium for 1 h. For experimental details and calibration procedures see Moolenaar *et al.* (1984, 1986).

(Fig. 3). At 1 μM -bradykinin, the rise in $[\text{Ca}^{2+}]_i$ occurs without a detectable lag period (< 1 s) and reaches a peak within 10–20 s, representing a 4–5-fold increase in $[\text{Ca}^{2+}]_i$. The rise in $[\text{Ca}^{2+}]_i$ is transient, decreasing to the resting level within 1–2 min of bradykinin addition.

When extracellular Ca^{2+} is removed by addition of 2 mM-EGTA the bradykinin-induced $[\text{Ca}^{2+}]_i$ transient in A431 cells is only slightly attenuated ($< 20\%$) (results not shown), indicating that the Ca^{2+} signal is due to the release of Ca^{2+} from intracellular stores.

Separation of inositol phosphates

The time-course of formation and degradation of [^3H]inositol phosphates extracted from A431 cells was analysed by means of h.p.l.c. anion-exchange chromatography. After eluting the column with a linear gradient of 0–1.5 M-ammonium formate/phosphoric acid (pH 3.7) at least ten peaks can be observed (Fig. 4). $\text{Ins}(1)\text{P}$, $\text{Ins}(1,4)\text{P}_2$ and $\text{Ins}(1,4,5)\text{P}_3$ were identified by adding purified ^3H -labelled inositol phosphates (Amersham) to the samples. Some variation in the basal levels of ^3H -labelled inositol phosphates was observed, particularly in InsP and InsP_2 (approx. 2-fold). As in Irvine *et al.* (1984), the peak coeluting with ATP and running close before $\text{Ins}(1,4,5)\text{P}_3$ is designated $\text{Ins}(1,3,4)\text{P}_3$. At increasing ionic strength one complex peak (at 0.85 M) and two single peaks (at 1.0 and 1.2 M respectively) were observed. By analogy with the data of Heslop *et al.* (1985), one of the two peaks collected in fractions 120–130 is presumed to represent $\text{Ins}(1,3,4,5)\text{P}_4$. The other two peaks, collected in fractions 140–150 and 163–170, are thought to be the higher phosphorylated inositol phosphates InsP_5 and InsP_6 respectively, as first described by Heslop *et al.* (1985). Additional elution with 3 M-ammonium formate did not release any further radioactivity from the column. In unstimulated A431 cells, the presumptive InsP_5 peak is remarkably high: the estimated [^3H] InsP_5 level is 2–3 times higher than that of InsP and represents about 40% of the total [^3H]inositol phosphates.

Time course of bradykinin-induced inositol phosphate formation

Measurements of inositol phosphates in the presence of 10 mM-LiCl demonstrate that a rapid production of inositol phosphates take place after addition of bradykinin (Figs. 1 and 2). H.p.l.c. analysis shows that the levels of the various inositol phosphates are markedly increased after stimulation with bradykinin for 30 s (Fig. 5). To investigate how the levels of the individual inositol phosphates are affected, we monitored the kinetics of their formation between 0 and 10 min after addition of 1 μM -bradykinin to A431 cells. There is an acute, approx. 10-fold increase in $\text{Ins}(1,4,5)\text{P}_3$ within 15 s, which then rapidly declines to prestimulatory levels over a period of 1–2 min (Fig. 6). The time course of bradykinin-induced $\text{Ins}(1,4,5)\text{P}_3$ formation closely parallels the transient rise in $[\text{Ca}^{2+}]_i$ in bradykinin-stimulated A431 cells (cf. Fig. 3).

Bradykinin also evokes a dramatic increase in the levels of InsP_2 and InsP_4 , with maximum levels being reached just a few seconds after the $\text{Ins}(1,4,5)\text{P}_3$ peak (Fig. 6). Unlike the increase in $\text{Ins}(1,4,5)\text{P}_3$, the formation of InsP_4 (about 10 times the basal level) is not transient, but is sustained over a much longer period; an approx. 6-fold stimulation is still measured 10 min after bradykinin addition. Fig. 5 shows that the increase in InsP_2 (approx. 4-fold) declines slowly and reaches basal levels after 2–10 min.

As with other stimulated cells, the accumulation of $\text{Ins}(1,3,4)\text{P}_3$ shows a distinct delay of approx. 15 s (Fig.

Fig. 4. H.p.l.c. elution profile of inositol phosphates

Inositol phosphates from prelabelled, unstimulated A431 cells were analysed by h.p.l.c. anion-exchange chromatography as described under 'Materials and methods'. The elution profile from the column represents the ^3H radioactivity in the fractions (fraction size $250\ \mu\text{l}$). A continuous trace, with the data points omitted, is represented for clarity. The elution of the adenine nucleotide markers AMP, ADP and ATP, visualized by their absorption at 254 nm, and of $[^3\text{H}]\text{Ins}(1)\text{P}$, $[^3\text{H}]\text{Ins}(1,4)\text{P}_2$ and $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ standards are indicated. The broken line represents the linear ammonium formate gradient (0–1.5 M). The peak immediately following the $\text{Ins}(1,4)\text{P}_2$ peak has not been identified and perhaps represents another InsP_2 isomer.

Fig. 5. H.p.l.c. elution profile of bradykinin-stimulated A431 cells

$[^3\text{H}]\text{Inositol}$ -prelabelled cells were stimulated with $1\ \mu\text{M}$ -bradykinin for 30 s in the absence of LiCl. Incubations were terminated and inositol phosphates analysed by h.p.l.c. as described under 'Materials and methods'. InsP and InsP_2 were collected in 0.5 min fractions ($650\ \mu\text{l}$), $\text{Ins}(1,3,4)\text{P}_3$ and $\text{Ins}(1,4,5)\text{P}_3$ in 0.1 min fractions ($125\ \mu\text{l}$) and InsP_4 , InsP_5 and InsP_6 in 1 min fractions ($1.25\ \text{ml}$). The elution profile represents the ^3H radioactivity in the fractions of bradykinin-stimulated (broken line) and control (continuous line) cells.

Fig. 6. Time course of bradykinin-induced inositol phosphate metabolism

Prelabelled cultures were incubated with $1 \mu\text{M}$ -bradykinin for the indicated times in the absence of LiCl. Termination of the incubations and analysis of the inositol phosphates by h.p.l.c. anion-exchange chromatography was performed as described under 'Materials and methods'. The data are all derived from one representative experiment ($n = 3$).

6). The $\text{Ins}(1,3,4)\text{P}_3$ isomer reaches a maximal stimulation of about 20 times the basal level after 60 s, about 30 s later than InsP_4 . As with InsP_4 formation, a 6-fold increase in $\text{Ins}(1,3,4)\text{P}_3$ is maintained over a period of at least 10 min.

Finally, a slow but massive increase in InsP is observed which reaches a peak after approx. 60 s and then slowly returns to its basal level. The levels of InsP_5 and InsP_6 are much less affected by bradykinin. Most notably, there is a small, but consistent, transient reduction in InsP_5 (Fig. 6). The mean decrease in InsP_5 at the 15 s time-point is $15 \pm 5\%$ ($n = 3$; $P < 0.05$ relative to the control).

Effects of pertussis toxin

In some cell types, there is evidence that a pertussis toxin-sensitive GTP-binding protein is involved in the coupling of hormone receptors to the hydrolysis of phosphoinositides (e.g. Nakamura & Ui, 1985; Volpi *et al.*, 1985). To test the effects of this bacterial toxin on bradykinin-stimulated inositol phosphate formation, the cells were incubated for 2 h in a medium containing 200 ng of pertussis toxin/ml. Fig. 7 clearly shows that neither the basal nor the bradykinin-induced (5 nM) inositol phosphate levels are significantly affected by pertussis toxin, although prolonged treatment (20 h)

Fig. 7. Effects of pertussis toxin on bradykinin-mediated inositol phosphate production

A431 cells were treated with (hatched bars) or without (open bars) pertussis toxin (200 ng/ml) for 2 h. The cultures were stimulated with 5 nM- or 1 μ M-bradykinin for 10 min in the presence of 10 mM-LiCl. The incubations were terminated and an inositol phosphate fraction was prepared as described under 'Materials and methods'. Data are expressed as means $\pm$ S.E.M. for three determinations. The asterisk indicates a significant difference ($P < 0.01$) between cultures treated with pertussis toxin and untreated controls.

reduced both levels by approx. 30%, without changing the relative stimulation (results not shown). Compared with untreated cells, the stimulation by a saturating concentration bradykinin (1 μ M) is significantly lower in cells treated with pertussis toxin (Fig. 7).

DISCUSSION

Our results provide the first evidence that the neuropeptide bradykinin stimulates inositol phosphate formation and raises free Ca^{2+} in human A431 cells, apparently through interaction with a specific membrane receptor, as previously reported for other cell systems (Pidikiti *et al.*, 1985; Yano *et al.*, 1985; Derian & Moskowitz, 1986). While the A431 cell line has been widely exploited for EGF-receptor studies, its strong responsiveness to bradykinin is a useful and previously unsuspected property of this cell line.

The major aim of the present study was to unravel the complex metabolism of inositol phosphates in A431 cells and to monitor the kinetics of their formation in response to bradykinin.

Separation of the inositol phosphates by h.p.l.c. anion exchange chromatography demonstrates that, in addition to the identified $\text{Ins}(1)P$, $\text{Ins}(1,4)P_2$ and $\text{Ins}(1,4,5)P_3$,

several other putative inositol phosphates are present in A431 cells. From their ionophoretic mobilities, and from previous analyses in other cells (Irvine *et al.*, 1985; Heslop *et al.*, 1985) we identify them as $\text{Ins}(1,3,4)P_3$, $\text{Ins}P_4$, $\text{Ins}P_5$ and $\text{Ins}P_6$; however, our identification of the higher phosphorylated forms of inositol is as yet preliminary in that radiolabelled markers of these inositol phosphates are not available. It is worth mentioning that, by using a weakly basic anion-exchange column, we were able to elute all the inositol phosphate peaks at a relatively low ionic strength (linear gradient to 1.5 M-ammonium formate); nevertheless, we obtained a perfect separation of the various peaks comparable with the elution pattern described by Heslop *et al.* (1985) who used a gradient to 3.4 M.

The height of the putative $\text{Ins}P_5$ peak (> 40% of the total ^3H radioactivity) is rather surprising. In the only previously published report of $\text{Ins}P_5$ and $\text{Ins}P_6$ in mammalian cells, Heslop *et al.* (1985) found that the amount of $\text{Ins}P_5$ in GH_4 cells is only slightly higher than that of $\text{Ins}P_4$ and $\text{Ins}P_6$. The function and metabolism of $\text{Ins}P_5$ and $\text{Ins}P_6$ in mammalian cells are unknown, although it is clear that their concentrations do not change dramatically upon hormonal stimulation of cells, as shown here for bradykinin in A431 cells (Fig. 6) and as earlier reported for TRH in GH_4 cells (Heslop *et al.*, 1985).

The bradykinin-induced alterations in the levels of $\text{Ins}(1,4,5)P_3$ coincide perfectly with changes in $[\text{Ca}^{2+}]_i$. Both parameters are stimulated within seconds after addition of bradykinin, and the $\text{Ins}(1,4,5)P_3$ peak slightly precedes the Ca^{2+} peak. This close correlation between the production of $\text{Ins}(1,4,5)P_3$ and intracellular calcium mobilization confirms the notion that $\text{Ins}(1,4,5)P_3$ is the second messenger for Ca^{2+} release from the endoplasmic reticulum (Berridge & Irvine, 1984).

Our kinetic studies allow a clear temporal separation of the formation of $\text{Ins}(1,4,5)P_3$, $\text{Ins}P_4$ and $\text{Ins}(1,3,4)P_3$ in A431 cells and indicate that part of the $\text{Ins}(1,4,5)P_3$ formed is phosphorylated to $\text{Ins}P_4$, followed by dephosphorylation to $\text{Ins}(1,3,4)P_3$. This precursor-product relationship, with $\text{Ins}P_4$ as intermediate, is entirely consistent with the view proposed by Batty *et al.* (1985) and further supported by the presence of $\text{Ins}(1,4,5)P_3$ kinase in various mammalian tissues (Irvine *et al.*, 1986). Recently this pathway has also been demonstrated in isolated rat hepatocytes (Hansen *et al.*, 1986).

The rapid formation of $\text{Ins}(1,3,4)P_3$ and $\text{Ins}P_4$ supports the proposal that, like $\text{Ins}(1,4,5)P_3$, these inositol phosphates could function as second messengers. However, whatever their possible biological functions, the kinetics of their formation argues against a role in calcium mobilization in stimulated cells. Thus, while the Ca^{2+} signal is initiated without a detectable lag period (< 1 s) and reaches a peak within 20–30 s, the formation of both $\text{Ins}P_4$ and $\text{Ins}(1,3,4)P_3$ is initiated only after a lag period of at least 15 s and reaches a peak at 30–60 s, when the Ca^{2+} signal is already declining. Furthermore, the increases in $\text{Ins}P_4$ and $\text{Ins}(1,3,4)P_3$ levels are much less transient and outlast the $[\text{Ca}^{2+}]_i$ signal by more than 10 min.

We note that, while $\text{Ins}P$ has returned to almost basal levels after 2 min, the levels of $\text{Ins}P_2$, $\text{Ins}(1,3,4)P_3$ and $\text{Ins}P_4$ are still elevated. The amount of $\text{Ins}P_2$ declines

slowly, reaching basal levels after approx. 10 min, whereas $\text{Ins}(1,3,4)\text{P}_3$ and InsP_4 levels remain elevated. This suggests that a new steady state for $\text{Ins}(1,3,4)\text{P}_3$ and InsP_4 has been reached at this time point. However, it is preliminary to draw definite conclusions as long as the nature of the rate-limiting steps in the metabolic degradation of InsP_4 remain to be elucidated.

The bradykinin response is hardly affected by pretreatment with pertussis toxin, indicating that the coupling between the receptor and the phosphodiesterase is not mediated by a pertussis toxin-sensitive GTP-binding protein. The small effects of pertussis toxin on both basal and agonist-stimulated inositol phosphate levels are perhaps attributable to an increase in cellular cyclic AMP levels (Takai *et al.*, 1982; Sano *et al.*, 1983).

In the continuous presence of bradykinin, a saturation of inositol phosphate production occurs within 30–60 s (Fig. 6). One possibility to explain this saturation phenomenon is a rapid depletion of the bradykinin-sensitive PtdInsP_2 pool. A second possibility, for which there is some indirect support, is that the receptor-mediated phosphoinositide hydrolysis is under inhibitory control, perhaps through diacylglycerol-activated protein kinase C (cf. Brock *et al.*, 1985; Orellana *et al.*, 1985; Leeb-Lundberg *et al.*, 1985).

Since our preliminary experiments indicate that bradykinin is a mitogen for A431 cells, as it is for fibroblasts (Owen & Villereal, 1983; Straus & Pang, 1984), and induces oncogene expression (W. Kruijen & W. H. Moolenaar, unpublished work), the present results raise the interesting possibility that $\text{Ins}(1,3,4)\text{P}_3$ and/or InsP_4 may somehow contribute to mitogenic signalling. In this respect it is noteworthy that exogenously added Li^+ causes a pronounced accumulation of $\text{Ins}(1,3,4)\text{P}_3$, at least in pancreatic acinar cells (Burgess *et al.*, 1985), while the stimulation of DNA synthesis by Li^+ in various cell types is well documented (Hori & Oka, 1979; Ptashne *et al.*, 1980; Rybak & Stockdale, 1981). Further experiments are needed to investigate the relationship between Li^+ -induced inositol phosphate accumulation and cell proliferation.

Now that the effects of bradykinin on inositol phosphate formation in A431 cells have been examined in detail, it will be of great interest to investigate the action of EGF on inositol phosphate metabolism and to compare it with that of bradykinin.

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