Differential mechanisms of inositol phosphate generation at the receptors for bombesin and platelet-derived growth factor

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We investigated the mechanism(s) whereby activation of a growth-factor receptor typically endowed with tyrosine kinase activity, such as the platelet-derived growth factor (PDGF) receptor, triggers phosphoinositide hydrolysis. In Swiss 3T3 cells permeabilized with streptolysin O, an analogue of GTP, guanosine 5'-[γ -thio]triphosphate, was found to potentiate the coupling of the bombesin receptor to phospholipase C. In contrast, the activation of the enzyme by PDGF occurred in a GTP-independent manner. Moreover, the inactive analogue of GTP, guanosine 5'-[β -thio]diphosphate, significantly inhibited the bombesin-induced InsP_3 generation, whereas it did not decrease the same effect when stimulated by PDGF.

INTRODUCTION

Mitogens can be divided in two groups, based on the different transduction mechanisms triggered by receptor activation. The first group includes classical growth factors such as PDGF, EGF and insulin, whose receptors possess an intrinsic ligand-sensitive protein tyrosine kinase activity. The tyrosine kinase receptors can be considered as transmembrane enzymes whose activity is regulated by ligand binding to the extracellular domain [1,2]. The second group of mitogens includes factors previously known as hormones, neuropeptides and even enzymes such as bombesin, bradykinin, vasopressin and thrombin. All these peptides interact with surface receptors coupled across the plasma membrane to a metabolic reaction, the hydrolysis by a specific phosphodiesterase (phospholipase C) of membrane phosphoinositides, in particular phosphatidylinositol 4,5-bisphosphate [3,4]. One of the two second messengers generated by this reaction, inositol 1,4,5-trisphosphate (InsP_3), regulates Ca^{2+} mobilization from intracellular stores; the other, diacylglycerol, is the physiological activator of protein kinase C. By analogy with the adenylate cyclase system, a coupling protein which binds and hydrolyses GTP (G protein) is believed to be physically and functionally interposed between receptors and phospholipase C [5].

In addition to tyrosine kinase stimulation, we and others have previously shown that PDGF and EGF also promote PI hydrolysis with intracellular Ca^{2+} increase and protein kinase C activation in Swiss 3T3 and A431 cells [6–9].

Only very recently, the coupling of the tyrosine kinase receptors to phospholipase C has been considered, with contrasting results [10,11]. We decided to investigate the molecular mechanism(s) whereby in permeable Swiss 3T3 cells the PDGF receptor (which typically possesses a tyrosine kinase activity) and the bombesin receptor

(which classically activates PI hydrolysis [12]) become coupled to phospholipase C.

MATERIALS AND METHODS

Materials

Swiss 3T3 fibroblasts were grown at 37 °C in Dulbecco's medium containing 10% (v/v) fetal-calf serum supplemented with penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). All the media and reagents for cell culture were purchased from Flow Laboratories. Ethidium bromide, bombesin and the various nucleotides were purchased from Sigma. PDGF purified from outdated human platelets by chromatography on CM-cellulose and Blue Sepharose as described in ref. [6] was kindly given by Dr. E. Sturani and Dr. R. Zippel. *myo*-[^3H]inositol (14 Ci/mmol) was obtained from Amersham, Dowex 1X8 (formate form) from Bio-Rad and streptolysin O from Wellcome Diagnostic.

Labelling and permeabilization of cells

Swiss 3T3 cells were subcultured in 100 mm-diam. Petri dishes and incubated for 24 h in inositol-free basal Eagle's medium supplemented with 5% dialysed fetal-bovine serum and containing *myo*-[^3H]inositol (2 $\mu\text{Ci}/\text{ml}$). On the day of the experiment, monolayers were washed in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline (130 mM-NaCl/0.2 mM-EDTA/3.3 mM- NaH_2PO_4 /6.7 mM- Na_2HPO_4) and mildly treated with trypsin. Cells were then suspended in the same basal Eagle's medium/5% serum in which they had been incubated before trypsin treatment and allowed to recover for 2 h at 37 °C in an oscillating bath. After three washes in Krebs buffer [containing (mM): NaCl 125; KCl 5; KH_2PO_4 and MgSO_4 1.2; CaCl_2 2; Hepes, pH 7.4, 25; glucose 6] the cells were washed once and then suspended in potassium glutamate buffer (0.12 M-potassium glutamate, 0.5 mg of bovine serum albumin/ml, 0.02 M-

Abbreviations used: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; PI, phosphoinositide; GTP[S], guanosine 5'-[γ -thio]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate.

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potassium acetate, 0.02 M-Pipes, 0.005 M-glucose, pH 6.8 at 37 °C). Then 250 μ l of cell suspension was transferred to an equal volume of buffer containing (final concns.): streptolysin O (0.4 unit/ml), CaEGTA buffer prepared to maintain free Ca^{2+} at 100 nM, MgCl_2 3 mM, ATP 2 mM. Cells were kept at 37 °C for 5 min.

The permeability of the plasma membrane of the cells was assessed by nuclear staining by the fluorescent probe ethidium bromide and measurement of the released lactate dehydrogenase recovered in the supernatant [13].

Inositol phosphate determination

After incubation for 5 min with the permeabilization buffer, the cells were treated with the various stimuli for a further 5 min and then rapidly sedimented by centrifugation. The supernatants were adjusted to pH 7.4 with sodium tetraborate, loaded directly on to Dowex columns and eluted as described by Berridge *et al.* [14].

RESULTS AND DISCUSSION

Fig. 1(a) shows the effect of increasing concentrations of the hydrolysis-resistant GTP analogue GTP[S] on the InsP_3 accumulation in permeable Swiss 3T3 cells in the absence and in the presence of a saturating dose of bombesin. In the absence of the peptide, GTP[S], known to activate G protein even without extracellular signals, stimulated the formation of InsP_3 , with a half-maximal effect at a concentration of about 2 μM . GTP[S] had no effect on InsP_3 accumulation in intact cells (results not shown). In the presence of a maximal dose of bombesin, the GTP[S] dose/response curve for InsP_3 release was shifted to the left, resulting in a decrease in the half-maximal effective concentration (EC_{50}) from 2 μM to about 0.2 μM .

The concentration-dependence for bombesin-stimulated InsP_3 generation is shown in Fig. 1(b). The peptide half-maximal effect was at a concentration of about

2 nM. A significant potentiation by 0.1 μM -GTP[S] was observed over the whole range of bombesin concentrations, whereas the EC_{50} of the mitogen was not altered in the presence of the nucleotide.

Among the various nucleotides tested (GTP, GTP[S], UTP, adenosine 5'-[β - γ -imido]triphosphate), only GTP and GTP[S] enhanced the bombesin-stimulated InsP_3 accumulation (results not shown). Taken together, these results appear consistent with a coupling of the bombesin receptor to phospholipase C via a G protein. It should be noted that in the permeable-cell system bombesin stimulated the accumulation of InsP_3 even in the absence of added GTP[S]. It is possible that permeable cells retain residual endogenous GTP levels that are sufficient to support the receptor-triggered response.

PDGF, in the absence of GTP[S], significantly stimulated the hydrolysis of PI in permeable Swiss 3T3 cells [Fig. 2(b)]. The dose/response curve was comparable with that observed in intact cells (results not shown). At variance with bombesin, however, the PDGF stimulatory effect on InsP_3 accumulation was not affected by the presence of 0.1 μM -GTP[S] (Fig. 2b). Moreover, a saturating concentration of PDGF did not affect the GTP[S] sensitivity of InsP_3 release (Fig. 2a).

The experiments shown in Fig. 2 suggest that a stimulatory G protein is not involved in the activation of phospholipase C by PDGF. This suggestion is strengthened by the data shown in Fig. 3. The GDP analogue GDP[S], known to inhibit the activation of G proteins, significantly decreased the bombesin-stimulated InsP_3 accumulation, with a maximal inhibition of 65% occurring at 100 μM -GDP[S]. A comparable dose/response curve for the GDP[S]-inhibitory effect on the bombesin-induced phosphorylation of an M_r -80000 protein in permeable Swiss 3T3 cells was obtained by Erusalimsky *et al.* [15]. In contrast, GDP[S] did not decrease the InsP_3 accumulation by PDGF. On the contrary, the GDP[S] pretreatment induced a slight,

Fig. 1. Dose/response curve for stimulation of $[\text{H}]\text{InsP}_3$ production by GTP[S] and bombesin in Swiss 3T3 cells

Permeabilized Swiss 3T3 cells were incubated for 5 min in potassium glutamate buffer containing MgCl_2 (3 mM) and ATP (2 mM) in CaEGTA buffer (free $[\text{Ca}^{2+}] = 100 \text{ nM}$) and in (a) increasing concentrations of GTP[S] in the absence (○) or in the presence (●) of bombesin (200 nM), or in (b) increasing concentrations of bombesin in the absence (○) or in the presence (●) of GTP[S] (0.1 μM). Results are expressed as differences ($\Delta \text{c.p.m.}$) over respective basal values [i.e. in (a) buffer alone or bombesin; in (b) buffer alone or GTP[S]] of the radioactivity in the $[\text{H}]\text{InsP}_3$ fraction. Each point represents the mean $\pm$ S.E.M. of three replicate determinations. A two-way ANOVA and a Tuckey test revealed a significant difference ($P < 0.01$) for both the shift in the GTP[S] sensitivity of InsP_3 release in the presence of bombesin (a) and the potentiation by GTP[S] of the bombesin response (b).

Fig. 2. Dose/response curve for stimulation of $[^3\text{H}]\text{InsP}_3$ production by GTP[S] and PDGF in Swiss 3T3 cells

Permeabilized Swiss 3T3 cells were incubated for 5 min in potassium glutamate buffer containing MgCl_2 (3 mM) and ATP (2 mM) in CaEGTA buffer (free $[\text{Ca}^{2+}] = 100 \text{ nM}$) and in (a) increasing concentrations of GTP[S] in the absence ($\circ$) or in the presence ($\bullet$) of PDGF (500 ng/ml), or in (b) increasing concentrations of PDGF in the absence ($\circ$) or in the presence ($\bullet$) of GTP[S] (0.1 μM). Results are expressed as differences ($\Delta\text{c.p.m.}$) over respective basal values (i.e. in (a) buffer alone or PDGF; in (b) buffer alone or GTP[S]) of the radioactivity in the $[^3\text{H}]\text{InsP}_3$ fraction.

Fig. 3. Dose/response curve for the GDP[S] effect on bombesin- and PDGF-stimulated accumulation of $[^3\text{H}]\text{InsP}_3$

Permeabilized Swiss 3T3 cells in potassium glutamate buffer, containing MgCl_2 (3 mM) and ATP (2 mM) in CaEGTA buffer (free $[\text{Ca}^{2+}] = 100 \text{ nM}$), were preincubated for 5 min with increasing concentrations of GDP[S] and then for a further 5 min with PDGF (500 ng/ml) ($\circ$) or bombesin (200 nM) ($\bullet$). Data, expressed as a percentage of the maximal InsP_3 release induced by bombesin and PDGF in the absence of GDP[S], represent means $\pm$ S.E.M. of three replicate determinations.

although not statistically significant, increase in the InsP_3 formed after stimulation with PDGF. Similar observations were recently made also by Hasegawa-Sasaki *et al.* [10], who suggest the existence of an

inhibitory G protein that might control the PDGF action on PI hydrolysis.

Taken together, these and the previous data suggest that a stimulatory G protein does indeed regulate the coupling of the bombesin receptor to phospholipase C, whereas the activation of the enzyme by PDGF occurs via a GTP-independent mechanism. On the basis of different experimental evidence, Blakeley *et al.* [16] also recently suggested that the PDGF receptor coupling to phospholipase C is fundamentally different from the coupling mechanism utilized by bombesin.

The recent elucidation of the PDGF (and EGF) receptor structure by molecular cloning argues against the possibility of interaction with phospholipase C via a G protein. In fact, the presence of a single transmembrane domain distinguishes the tyrosine kinase receptors from the group of receptors classically coupled to phospholipase C, which contain seven hydrophobic domains spanning the plasma membrane and a cytoplasmic region devoted to the interaction with target G proteins [17].

If a stimulatory G protein is not directly involved in the activation of phospholipase C by PDGF, the possibility that the receptor tyrosine kinase activity is responsible for the enzyme activation should be considered. Recent evidence showed that, in cells expressing tyrosine kinase-defective mutants of the PDGF and EGF receptors, the turnover of inositol phospholipids and the Ca^{2+} increase are abolished [18]. This result indicates that the tyrosine kinase activity is required for PDGF- and EGF-stimulated PI hydrolysis to occur. Moreover, it has recently been suggested that phospholipase C is a substrate for the EGF-stimulated tyrosine phosphorylation, and that this tyrosine phosphorylation might have consequences on the activity of the enzyme [19,20].

In an attempt to demonstrate the involvement of tyrosine kinase activity in the stimulation of PI hydrolysis by PDGF, the effect of bombesin and PDGF on InsP_3 accumulation was measured in the presence and in the

absence of added ATP, i.e. under conditions in which the phosphorylation reaction was either possible or inhibited. In permeable Swiss 3T3 cells we found that the absence of ATP totally inhibited the PDGF-stimulated, but not the bombesin-stimulated, accumulation of InsP_3 (results not shown). From these results, however, we cannot conclude that a phosphorylating step is involved in the PDGF action on PI hydrolysis. In fact, in the absence of ATP, even the accumulation of InsP_3 induced by bombesin was decreased by 50%. It is likely that ATP augmentation of bombesin-stimulated InsP_3 generation was due to the replenishment of the PI pool mediated by PI kinase [21], whereas the limited reaction observed in the absence of ATP represented the activated hydrolysis of pre-existing pools. Therefore, in the absence of ATP the effect of PDGF, which is a stimulus weaker than bombesin, might have been too small to be experimentally detected.

In conclusion, from the data presented, two classes of receptors stimulating PI hydrolysis can be distinguished. One class, represented here by the bombesin receptor, requires a G protein to activate phospholipase C, whereas a second class, represented by the PDGF tyrosine kinase receptor, stimulates PI hydrolysis via a different mechanism(s) which, however, has not been defined yet. Further experimental work is needed to clarify how the coupling of tyrosine kinase receptors to phospholipase C occurs.

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