Effects of pertussis toxin on growth factor-stimulated inositol phosphate formation and DNA synthesis in Swiss 3T3 cells

Colin W. TAYLOR,*† Diane M. BLAKELEY,† Anthony N. CORPS,† Michael J. BERRIDGE* and Kenneth D. BROWN†

*AFRC Unit of Insect Neurophysiology and Pharmacology, Department of Zoology, Downing Street, Cambridge CB2 3EJ, and †AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, U.K.

We have compared the effects of pretreatment of Swiss 3T3 cell with pertussis toxin on the stimulation of DNA synthesis and phosphoinositide hydrolysis in response to a wide variety of mitogens. The toxin substantially inhibited the stimulation of DNA synthesis in response to a phorbol ester or various peptide and polypeptide growth factors irrespective of their ability to activate phosphoinositidase C. Production of inositol phosphates in response to platelet-derived growth factor, fibroblast growth factor and prostaglandin $F_{2\alpha}$ were unaffected by the toxin while bombesin- and vasopressin-stimulated formation of inositol phosphates were inhibited by only 27 and 23 % respectively. These results argue against a major role for a pertussis toxin-sensitive G protein in coupling any of these mitogen receptors to activation of a phosphoinositidase C. Furthermore, the results suggest that the widespread inhibitory effects of pertussis toxin on mitogen-stimulated DNA synthesis may be unrelated to the toxin's limited actions on phosphoinositide hydrolysis.

INTRODUCTION

A variety of purified mitogens stimulate quiescent Swiss 3T3 cells to re-enter the cell cycle. Each mitogen binds to specific high-affinity cell-surface receptors and thereby initiates a sequence of events that culminates in DNA synthesis and mitosis. The links between occupation of the cell-surface receptors and nuclear events remain undefined. Here we concentrate on one of the early events that follows activation of certain receptors for growth factors, namely, stimulation of phosphoinositidase C with consequent formation of two intracellular messengers diacylglycerol and inositol 1,4,5-trisphosphate [1–3].

Bombesin (or the related mammalian homologue, gastrin-releasing peptide) and platelet-derived growth factor (PDGF) are potent stimulators of DNA synthesis in quiescent cultures of Swiss 3T3 cells [4] and both mitogens induce a rapid activation of phosphoinositidase C in these cells [5–7]. Whereas the PDGF receptor has an integral tyrosine kinase activity [8], a feature shared with receptors for several other growth factors [9], the bombesin receptor appears to lack such activity [10]. The only known transduction mechanism associated with the bombesin receptor is, therefore, its ability to stimulate phosphoinositidase C [5,7] raising the interesting possibility that such activation alone may be sufficient to stimulate proliferation in Swiss 3T3 cells.

In some cells, most notably those of haematopoietic origin, treatment with pertussis toxin blocks agonist-mediated stimulation of phosphoinositidase C [11]. By analogy with the effects of the toxin on G_i (the G protein that couples receptors to the inhibition of adenylate cyclase), pertussis toxin is thought to uncouple certain receptors from phosphoinositidase C by causing ADP-ribosylation of a G protein proposed to mediate that

coupling [12–14]. Here we examine the effects of pertussis toxin on the ability of several agonists to stimulate phosphoinositidase C and DNA synthesis in Swiss 3T3 cells. Our aims were to determine firstly whether pertussis toxin could reveal differences in the mechanisms whereby different receptors are coupled to phosphoinositidase C, and secondly to see whether we could dissociate the stimulation of phosphoinositidase C from the stimulation of DNA synthesis.

MATERIALS AND METHODS

Stock cultures of Swiss 3T3 cells were maintained as previously described [15]. Cells were seeded into 24-well culture trays (Nunc) in Dulbecco's modified Eagle's medium supplemented with 10 % calf serum and used 7 days later when they were confluent and quiescent. Pertussin toxin was added directly to the growth medium for the times indicated in the Figure and Table legends. For measurement of DNA synthesis, cells were rinsed twice with serum-free Dulbecco's modified Eagle's medium and 2 ml of this medium containing [3 H]thymidine (1 μ Ci/ml, 1 μ M) was added per dish. Mitogens were added at the concentrations indicated in the legends and the cells were incubated at 37 °C for 40 h. The cells were washed, extracted, and incorporated [3 H]thymidine was measured as described previously [15]. For measurement of inositol phosphates, parallel cell cultures, prepared together with those used in the DNA synthesis assays, were labelled for the final 70 h of growth by the addition of *myo*-[3 H]inositol (10 μ Ci/ml) directly to the growth medium. After labelling, the cells were washed twice in phosphate-buffered saline (pH 7.4) [7] and then incubated at 37 °C in 300 μ l of phosphate-buffered saline containing 10 mM-LiCl. After 10 min

Abbreviations used: FGF, basic fibroblast growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor.

† To whom correspondence should be addressed.

agonists were added and 5 min later reactions were stopped by addition of 600 μ l of ice-cold 4.5% perchloric acid. Inositol phosphates were allowed to extract for 30 min, the extract was removed and the wells were washed with $2 \times 300 \mu$ l of 3% perchloric acid. The cell extract and washes were combined, neutralized [16,17], and total [3 H]inositol phosphate (inositol mono-, bis-, tris- and tetrakis-phosphates) were eluted from anion-exchange columns [18] in 8 ml of 1.2 M-ammonium formate/0.1 M-formic acid.

After perchloric acid extraction, the cells were extensively washed with distilled water and finally with 100 mM-EDTA. Lipids were then deacylated in the wells by a simple modification of the method of Clarke & Dawson [19]. Briefly, 300 μ l of monomethylamine reagent was added to each well and the tray incubated at 53 °C for 30 min. After drying under vacuum, the deacylation products were dissolved in 1 ml of distilled water. The aqueous extract was washed in butan-1-ol/light petroleum (5:1, v/v) [19], dried, redissolved in distilled water and loaded on to anion-exchange columns for separation of the deacylated lipids [20].

Bombesin was obtained from Bachem (Saffron Walden, U.K.). Radiochemicals were from Amersham International. Basic fibroblast growth factor (FGF) was prepared as described [21] and appeared to be > 90% pure as assessed by silver-stained SDS/polyacrylamide-gel electrophoresis. The protein concentration of the preparation was too low for determination by routine assay and was not measured; 2 μ l/ml of the preparation was maximally active in stimulating DNA synthesis in Swiss 3T3 cells. PDGF was a gift from Dr. C. H. Heldin (Uppsala, Sweden). AG 1X-8 (200–400 mesh, formate form) anion-exchange resin was from Bio-Rad. Pertussis toxin was from List Biologicals (Campbell, CA, U.S.A.). All other reagents were from Sigma or Aldrich Chemical Company.

RESULTS AND DISCUSSION

Two recent reports have suggested that the stimulation of DNA synthesis in cultured fibroblasts by different mitogens may differ in susceptibility to pertussis toxin. In Swiss 3T3 cells, Letterio *et al.* [22] reported that pertussis toxin (5–10 mg/ml for 2–3 h) eliminated bombesin-stimulated DNA synthesis and mitosis without affecting the mitogenic effects of PDGF or a phorbol ester. In a Chinese hamster lung fibroblast line (CCL39) pertussis toxin (3–10 ng/ml for 4 h) substantially inhibited thrombin-stimulated DNA synthesis but not the responses to epidermal growth factor (EGF) or FGF [23]. In contrast with these results, we have found that even prolonged pretreatment of Swiss 3T3 cells with pertussis toxin (100 ng/ml for 65 h) reduces bombesin-stimulated DNA synthesis only to 62% of control (Table 1). This effect of pertussis toxin cannot be ascribed to a reduction in cellular binding of bombesin since we have found no reduction in the binding of 125 I-[Tyr⁴]bombesin to the toxin-treated cells. Indeed at saturating ligand concentrations (10 nM), there was actually a small increase (15%) in binding to the treated cells (results not shown).

In subsequent studies using other mitogens, we treated cells with pertussis toxin at 1 ng/ml for 48 h since that was sufficient to give the largest inhibition of bombesin-stimulated DNA synthesis and inositol phosphate formation (Tables 1 and 2). These experiments demonstrated

Table 1. Effects of different concentrations of pertussis toxin on bombesin-stimulated [3 H]thymidine incorporation and [3 H]inositol phosphate formation

Cells were treated with the indicated concentrations of pertussis toxin for 65 h before stimulation with bombesin (3 nM) and measurement of either [3 H]thymidine incorporation or total [3 H]inositol phosphate formation as described in the Materials and methods section. Results are means $\pm$ S.E.M. of triplicate determinations from a single experiment. Similar results were obtained in a second experiment.

Pertussis toxin (ng/ml)	[3 H]Inositol phosphate (c.p.m./well)		$10^{-3} \times$ [3 H]Thymidine incorporated (c.p.m./well)	
	Control	Bombesin	Control	Bombesin
0.0	422 $\pm$ 45	3726 $\pm$ 69	3.1 $\pm$ 0.5	67.5 $\pm$ 1.7
0.1	373 $\pm$ 50	3180 $\pm$ 62	7.0 $\pm$ 2.9	38.6 $\pm$ 2.6
1.0	350 $\pm$ 52	3033 $\pm$ 135	3.0 $\pm$ 1.6	41.7 $\pm$ 2.8
10.0	386 $\pm$ 76	2923 $\pm$ 20	1.7 $\pm$ 0.6	36.9 $\pm$ 4.1
100.0	338 $\pm$ 52	3047 $\pm$ 150	1.6 $\pm$ 0.3	41.9 $\pm$ 5.5

Table 2. Effects of duration of pertussis toxin treatment on bombesin-stimulated [3 H]thymidine incorporation and [3 H]inositol phosphate formation

Cells were treated with pertussis toxin (1 ng/ml) for the indicated times before stimulation with bombesin (3 nM) and measurement of [3 H]thymidine incorporation or total [3 H]inositol phosphate formation as described in the Materials and methods section. Results are means $\pm$ S.E.M. of triplicate determinations from a single experiment. A second experiment gave similar results.

Time of exposure to pertussis toxin (h)	[3 H]Inositol phosphate (c.p.m./well)		$10^{-3} \times$ [3 H]Thymidine incorporated (c.p.m./well)	
	Control	Bombesin	Control	Bombesin
0	547 $\pm$ 49	3444 $\pm$ 111	4.5 $\pm$ 2.3	65.6 $\pm$ 2.9
5	553 $\pm$ 73	3052 $\pm$ 74	5.9 $\pm$ 1.5	64.9 $\pm$ 6.4
18	577 $\pm$ 125	2516 $\pm$ 113	5.0 $\pm$ 2.0	48.4 $\pm$ 6.8
48	443 $\pm$ 60	2793 $\pm$ 41	7.4 $\pm$ 5.7	33.4 $\pm$ 2.4
70	537 $\pm$ 42	2385 $\pm$ 100	2.1 $\pm$ 0.2	37.2 $\pm$ 1.4

that prior treatment with pertussis toxin similarly inhibited the ability of a variety of mitogens to stimulate DNA synthesis including those that had either no effect (12-*O*-tetradecanoylphorbol 13-acetate) or minimal effects (insulin and EGF) on phosphoinositide hydrolysis (Fig. 1). We were unable to assess the effects of fluoride on DNA synthesis since it was toxic in prolonged incubations.

The effects of pertussis toxin on mitogen-stimulated [3 H]inositol phosphate formation were complex: responses to the bombesin, vasopressin, and most notably NaF were inhibited, while the responses to PDGF, prostaglandin F_{2 α} and FGF were unaffected (Fig. 1*b*). At least some of the inhibitory effects may be accounted for by the small decrease in cell number (reduced to $92 \pm 2\%$

Fig. 1. Effects of pertussis toxin on $[{}^3\text{H}]$ thymidine incorporation and $[{}^3\text{H}]$ inositol phosphate formation in cells stimulated with various growth factors

Cells were treated with pertussis toxin (1 ng/ml) for 48 h before measurement of either $[{}^3\text{H}]$ thymidine incorporation (a) or $[{}^3\text{H}]$ inositol phosphate formation (b) in response to mitogens. PDGF (3.3 ng/ml), bombesin (3 nM), prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) (200 ng/ml), 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (100 ng/ml), vasopressin (30 nM), FGF (20 $\mu\text{g}/\text{ml}$), NaF (20 mM), EGF (10 ng/ml) and insulin (1 $\mu\text{g}/\text{ml}$). In both panels, open columns and cross-hatched columns represent cultures stimulated without or with pertussis toxin pretreatment respectively. Results are means $\pm$ S.E.M. of 6–18 determinations from between two and six experiments.

of control), the small loss in total phosphoinositides (reduced to $85 \pm 1\%$ of control), and the decrease in ratio of phosphatidylinositol 4,5-bisphosphate to total phosphoinositides (4.9% to 4.1%; Table 3) in the toxin-treated cells. However, this clearly is not a complete explanation since it cannot easily explain the differential effects of pertussis toxin on the stimulation of $[{}^3\text{H}]$ -inositol phosphate production by the various agonists. The stimulation of $[{}^3\text{H}]$ inositol phosphate formation by NaF, presumably as AlF_4^- , is most markedly inhibited by the toxin (Fig. 1b). This observation and a similar report [24] are surprising when compared with the effects of pertussis toxin on receptor-mediated inhibition of adenylate cyclase, where the coupling protein, G_i , is known to be covalently modified by the toxin. In that system, pertussis toxin uncouples receptors from adenylate cyclase without impairing interaction of the G protein with either guanine nucleotides or adenylate cyclase [25].

Table 3. Effects of pertussis toxin on $[{}^3\text{H}]$ phosphoinositides

Cells were incubated with or without pertussis toxin (1 ng/ml) for 48 h, and then washed and incubated for 20 min in phosphate-buffered saline containing 10 mM-LiCl (i.e. the conditions used for experiments involving measurements of $[{}^3\text{H}]$ inositol phosphate formation). Reactions were stopped and lipids were deacylated and separated as described in the Materials and methods section. Results are means $\pm$ S.E.M. of three independent experiments each with triplicate determination. Abbreviations: PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate.

Inositide	$[{}^3\text{H}]$ Phosphoinositides (% of total)	
	Control	Pertussis toxin-treated
PI	91.1 ± 1.9	93.0 ± 0.4
PIP	2.9 ± 0.3	2.5 ± 0.2
PIP_2	4.9 ± 0.5	4.1 ± 0.2

Similarly in neutrophils pertussis toxin has been reported to abolish stimulation of phosphoinositidase C by chemotactic peptide but not by fluoride which is thought to activate G proteins directly [26]. It therefore seems likely that the observed effect of pertussis toxin on fluoride-stimulated inositol phosphate formation may be unrelated to any effect on the G protein proposed to regulate phosphoinositidase C.

Since measurement of DNA synthesis required longer incubations after addition of mitogens than did measurement of $[{}^3\text{H}]$ inositol phosphate formation (48 h versus 15 min), we were concerned that a substrate for pertussis toxin that had been modified during pretreatment could be either resynthesized, or the modification reversed, during the subsequent long incubation in the absence of toxin. However, this seems unlikely since, when cells labelled with $[{}^3\text{H}]$ inositol were treated with pertussis toxin (1 ng/ml for 48 h) and then either immediately stimulated with mitogen or washed and incubated for a further 48 h without toxin before addition of mitogen, the effects of mitogens on the formation of $[{}^3\text{H}]$ inositol phosphates were similarly attenuated in each case (results not shown). Nor is there a general decline in cell responsiveness during the longer incubations since, although cells lost $[{}^3\text{H}]$ inositol-labelled lipids during the 48 h period without $[{}^3\text{H}]$ inositol, the magnitude of mitogen-stimulated $[{}^3\text{H}]$ inositol phosphate formation was similar when expressed relative to basal levels, whether measured immediately after treatment with pertussis toxin, or 48 h after its removal (results not shown). Together these results establish that the effects of pertussis toxin neither reverse during the 48 h following removal, nor lead to any further decline in cell responsiveness.

Recently published results [22,23,27] raise the interesting possibility that pertussis toxin very strongly inhibits the actions of only those mitogens whose principle or sole signal-transducing pathway involves phosphoinositide hydrolysis. Bombesin on Swiss 3T3 cells or thrombin on CCL39 fibroblasts might be provisionally placed in this category. In contrast, pertussis toxin was observed not to inhibit the actions of other mitogens (PDGF on 3T3 cells, EGF or FGF on CCL39

fibroblasts) which are known to have other or additional signal-transducing actions. Our results argue against this attractively simple interpretation since we have observed relatively small inhibitory effects of pertussis toxin on the actions of a wide range of Swiss 3T3 cell mitogens. Furthermore, we were unable to demonstrate dramatic inhibition of bombesin-mediated effects on the cells despite testing high concentrations of the toxin administered for long periods. From our results, we conclude that there is no convincing evidence to suggest that any of the receptors examined in this study requires direct interaction with a pertussis toxin-sensitive G protein to stimulate either phosphoinositidase C or DNA synthesis. An earlier study on mouse 3T3 cells [28] demonstrated complex effects of pertussis toxin: receptor coupling to phosphoinositidase C was unaffected but several later events such as arachidonic acid release and uptake of Ca^{2+} very inhibited. Recently, enhancement of cyclic AMP production in Swiss 3T3 cells by phorbol esters was shown to be inhibited by pertussis toxin [29]. These results emphasize the complexity of the toxin's effects on cellular physiology and suggest that such effects may underlie the inhibitory actions of the toxin seen in our experiments.

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