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Pertussis toxin inhibits thrombin-induced activation of phosphoinositide hydrolysis and Na^+/H^+ exchange in hamster fibroblasts

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Prior treatment with pertussis toxin of G_0-arrested hamster fibroblasts (CCL39) results in a dose-dependent inhibition of two early events of the mitogenic response elicited by α-thrombin: (i) accumulation of inositol phosphates in Li^+-treated cells, and (ii) activation of the Na^+/H^+ antiport, measured either by the amiloride-sensitive $^{22}Na^+$ influx or by the increase in intracellular pH. At 10^{-1} U/ml of α-thrombin, the maximal inhibition was ~50% for these two early cellular responses, but the pertussis toxin effect was more pronounced at lower thrombin concentrations. In contrast, pertussis toxin does not affect the Na^+/H^+ antiport activation induced by phorbol esters or EGF, the action of which is not mediated by the phosphoinositide-metabolizing pathway in CCL39 cells. Therefore, our data suggest the following. (i) A GTP-binding regulatory protein is probably involved in signal transduction between thrombin receptors and the phosphatidylinositol 4,5-bisphosphate-specific phospholipase C. This regulation does not seem to be exerted via modulations of cyclic AMP levels. (ii) The thrombin-induced activation of Na^+/H^+ antiport is, at least in part, mediated by the protein kinase C, as a consequence of stimulation of phosphatidylinositol turnover.

Key words: growth factors/Na^+/H^+ antiport/pertussis toxin/phosphoinositide breakdown/thrombin

Introduction

Addition of mitogens to resting cells rapidly triggers a complex sequence of events leading ultimately to reinitiation of DNA synthesis and cell division. Among the earliest biochemical changes induced by growth factors, a ubiquitous cellular response is the stimulation of an amiloride-sensitive Na^+/H^+ exchange (for recent reviews, see Leffert and Koch, 1985; Rozengurt, 1985; Pouysségur, 1985; Moolenaar, 1986). We previously reported that, in the Chinese hamster fibroblast line CCL39, activation of the Na^+/H^+ antiporter by α-thrombin results from an alkaline shift in the pH_i sensitivity of the exchanger (Paris and Pouysségur, 1984). This activation leads to cytoplasmic alkalinization (L'Allemain *et al.*, 1984b), which was shown to have a permissive role in the mitogenic response (Pouysségur *et al.*, 1985a, 1985b).

More recently, we observed that α-thrombin also induces in CCL39 cells a rapid activation of membrane polyphosphoinositide breakdown (L'Allemain *et al.*, in preparation). Receptor-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) appears to be an important mediator of cell activation through the generation of the second messengers inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (reviewed by Berridge, 1984; Berridge and Irvine, 1984; Nishizuka, 1984; Majerus *et al.*, 1985). Diacylglycerol is known to activate protein kinase C and tumor-promoting phorbol esters have been shown to act as a substitute for diacylglycerol and directly activate this protein kinase *in vitro* and *in vivo* (Castagna *et al.*, 1982; Nishizuka, 1984).

As phorbol esters or a synthetic diacylglycerol activate the Na^+/H^+ exchange in a variety of cell types, including fibroblasts (Dicker and Rozengurt, 1981; Moolenaar *et al.*, 1984; Hesketh *et al.*, 1985; Vara *et al.*, 1985; Pouysségur *et al.*, 1985a), lymphocytes (Rosoff *et al.*, 1984; Grinstein *et al.*, 1985), a leukemic cell line (Besterman and Cuatrecasas, 1984) and proliferating myoblasts (Vigne *et al.*, 1985), the growth factor-induced modification of the antiporter is likely to be, at least in part, mediated by protein kinase C as a consequence of activation of phosphatidylinositol turnover.

In CCL39 cells, persistent activation of both phosphoinositide breakdown and Na^+/H^+ exchange has been shown to require the continual presence of active α-thrombin at the cell surface (Van Obberghen-Schilling *et al.*, 1985), indicating a tight coupling of these two events with the membrane-bound signalling system. The exact nature of this system is still largely unknown. Recently, it has been proposed that the transduction mechanism between receptors and phospholipase C might involve a GTP-binding protein as in the adenylate cyclase system (Berridge and Irvine, 1984; Majerus *et al.*, 1985). Several recent observations support this idea. First, addition of GTP analogs to permeabilized mast cells (Cockroft and Gomperts, 1985), to neutrophil membranes (Cockroft and Gomperts, 1985; Smith *et al.*, 1985), or to salivary gland membranes (Litosch *et al.*, 1985) stimulates phosphoinositide breakdown in the presence of the appropriate effector. Second, pre-treatment of intact cells with pertussis toxin inhibits receptor-mediated activation of phosphatidylinositol turnover in mast cells (Nakamura and Ui, 1985), in neutrophils (Volpi *et al.*, 1985; Bradford and Rubin, 1985) and in a leukemic cell line (Brandt *et al.*, 1985). This bacterial toxin has been shown to catalyse the ADP-ribosylation of several GTP-binding proteins, such as the N_i protein responsible for adenylate cyclase inhibition (Ui, 1984; Jakobs *et al.*, 1984), the retinal transducin (Van Dop *et al.*, 1984; Manning *et al.*, 1984), and a so far unidentified 39/40-kd protein, termed G_0 by some, found in brain (Sternweis and Robishaw, 1984; Neer *et al.*, 1984), in fat cells (Malbon *et al.*, 1984) and in cardiac cells (Malbon *et al.*, 1985).

In this paper we examine the effect of pertussis toxin on the α-thrombin-induced activation of phosphoinositide breakdown and Na^+/H^+ exchange in CCL39 cells, in order to gain further insight into, first, the signal transduction mechanism and, second, into the causal relationship between these two early events of the mitogenic response.

Results

Pertussis toxin inhibits α-thrombin-induced inositol phospholipid breakdown

In G_0-arrested CCL39 cells, inositol phospholipid turnover is extremely slow. Treatment with Li^+, to inhibit the inositol 1-phosphatase (Berridge *et al.*, 1982), does not result in any significant

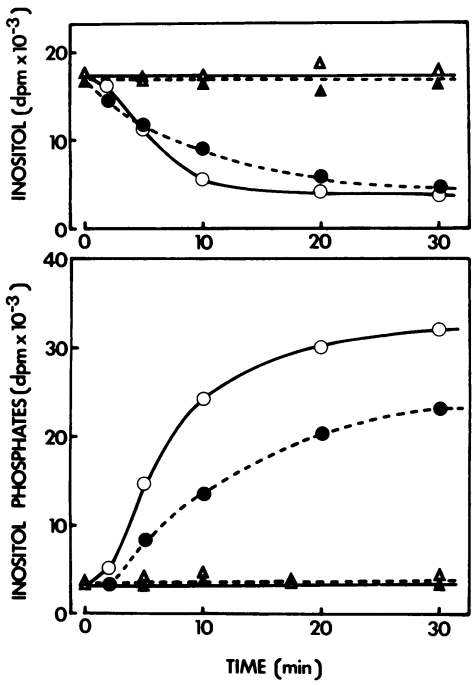

Fig. 1. Time-course for α-thrombin-induced phosphoinositide metabolism. Effect of pertussis toxin. [^{3}H]inositol-labeled cells treated with (●, ▲) or without (○, △) 1 μg/ml of pertussis toxin were incubated with (○, ●) or without (△, ▲) α-thrombin at 10^{-2} U/ml, in the presence of 20 mM Li^+. The incubation was terminated at various times after thrombin addition and the water-soluble cell extract was analysed for free [^{3}H]inositol (upper panel) and total [^{3}H]inositol phosphates (lower panel), as described under Materials and methods.

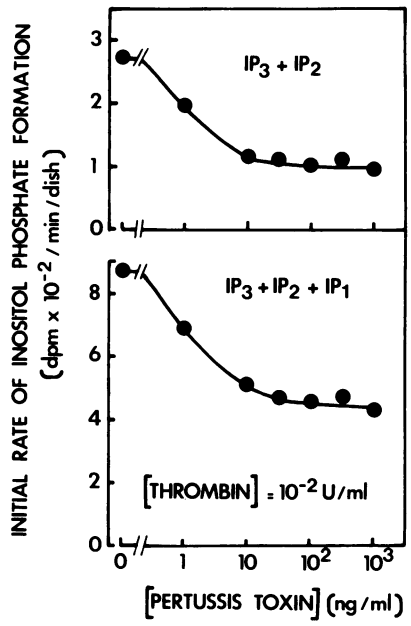

Fig. 2. Concentration-dependent inhibition by pertussis toxin of α-thrombin-induced [^{3}H]inositol phosphate generation. G_0-arrested CCL39 cells labeled with [^{3}H]inositol and exposed to increasing concentrations of pertussis toxin were incubated with 10^{-2} U/ml of α-thrombin, in the presence of 20 mM Li^+, as in Figure 1. The incubation was terminated after 2, 4 and 6 min and cell extracts were stepwise analysed for [^{3}H]inositol monophosphate (IP_1), bisphosphate (IP_2) and trisphosphate (IP_3). In the absence of thrombin, with or without toxin treatment, cell content in IP_1, IP_2 and IP_3 was 1930 ± 250, 390 ± 40 and 225 ± 15 c.p.m./dish, respectively (n = 4). Upon thrombin addition, IP_3 increased only transiently, due to rapid conversion to IP_2. In contrast, the combinations IP_3 + IP_2 and IP_3 + IP_2 + IP_1 were found to increase linearly over 6 min. Average initial rates of inositol phosphate accumulation over the basal level were determined from the three time points.

accumulation of water-soluble inositol phosphates over 30 min (Figure 1, lower panel). In contrast, addition of α-thrombin to Li^+-treated cells triggers inositol phosphate accumulation, reflecting activation of polyphosphoinositide breakdown. With low α-thrombin concentrations ($\leq 10^{-2}$ U/ml), a short lag was occasionally observed, as in Figure 1, but with higher concentrations, inositol phosphate formation could be detected within 5 s (L'Allemain *et al.*, in preparation). Pre-treatment of cells with 1 μg/ml of pertussis toxin for 3 h greatly reduces (by ~50%) the initial rate of thrombin-induced inositol phosphate generation, at 10^{-2} U/ml of α-thrombin (Figure 1). Addition of thrombin to Li^+-treated cells also causes a marked decrease in cellular free inositol (Figure 1, upper panel). Since the same observation was made when [^{3}H]inositol was maintained in the extracellular medium (not shown), this decrease is unlikely to be due to a thrombin-mediated stimulation of inositol efflux. Rather it reflects incorporation of inositol into phosphatidylinositol (PI), leading to polyphosphoinositide re-synthesis. Interestingly, pertussis toxin treatment affects this part of the PI cycle much less than the generation of inositol phosphates, particularly in the first 5 min after thrombin addition (Figure 1, upper panel). This observation suggests that PI re-synthesis is not simply controlled by PIP_2 concentration, but may also be directly stimulated upon thrombin addition.

Figure 2 shows that the effect of pertussis toxin on thrombin-induced inositol phosphate accumulation is concentration-dependent. Pre-treatment of cells with 1 ng/ml of pertussis toxin caused nearly half-maximal inhibition at 10^{-2} U/ml of α-thrombin. However, maximal inhibition did not exceed 50% for the formation of total inositol phosphates (inositol tris-, bis- and monophosphates: IP_3 + IP_2 + IP_1). No significant difference was observed after 2 and 3 h of toxin treatment, but no attempt was made with longer incubations. When the individual inositol phosphates were analysed, IP_3 appeared to accumulate very transiently (for ~2 min at 10^{-2} U/ml of α-thrombin). It was rapidly hydrolysed into IP_2, the accumulation of which was much more significant. It is noteworthy that the pertussis toxin effect was also observed on the formation of inositol tris- and bis-phosphates (IP_3 + IP_2 on Figure 2), with a similar dose-response. This result clearly indicates that the toxin treatment affects PIP_2 hydrolysis into IP_3 and diacylglycerol, rather than the direct cleavage of phosphatidylinositol into IP_1, a pathway stimulated by thrombin in platelets (Majerus *et al.*, 1985). The toxin-mediated inhibition of inositol phosphate formation appears slightly more pronounced when considering only the first two products IP_3 + IP_2, but this can be easily explained by the observation that the proportion of IP_2 converted to IP_1 increases when IP_2 formation is slowed down.

As shown in Figure 3, the initial rate of inositol phosphate formation increases with thrombin concentration. No saturation was observed up to 10 U/ml of α-thrombin. It must be noted that this is true only for initial rates. An apparent saturation was observed when inositol phosphate accumulation was measured after 3 min for all thrombin concentrations, probably due to a limitation in the substrate supply to phospholipase C. These kinetic properties will be detailed elsewhere (L'Allemain *et al.*, in preparation). The pertussis toxin effect was evident at all thrombin concentrations, though it was more important at the lowest ones. Inhibition varied from 60% to 30% in the range

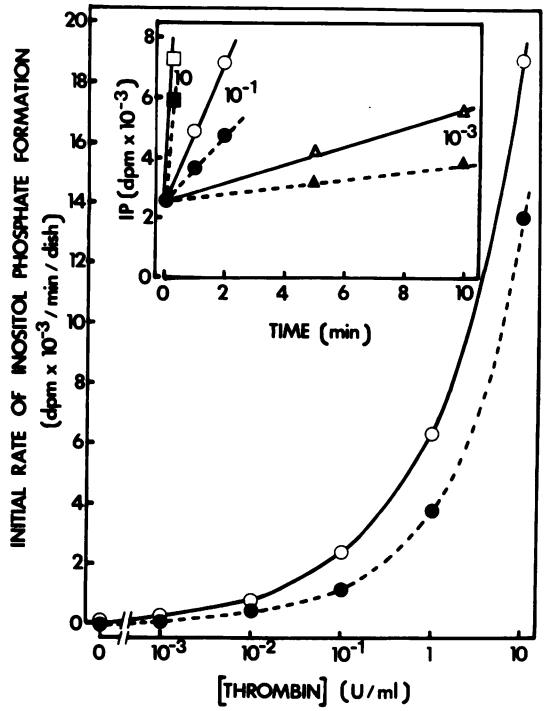

Fig. 3. Initiate rate of inositol phosphate formation as a function of α-thrombin concentration. Effect of pertussis toxin. Cells were treated (●, ▲, ■) or not (○, △, □) with 1 μg/ml of pertussis toxin and incubated with increasing concentrations of α-thrombin. As shown in the **inset** for three α-thrombin concentrations (10^{-3}, 10^{-1} and 10 U/ml), incubation times were chosen to allow determination of the initial rates of inositol phosphate formation: 5 and 10 min, 2 and 4 min, 1 and 2 min, 0.5 and 1 min, 15 and 30 s for 10^{-3}, 10^{-2}, 10^{-1}, 1 and 10 U/ml respectively. IP refers to total inositol phosphates (IP_3 + IP_2 + IP_1).

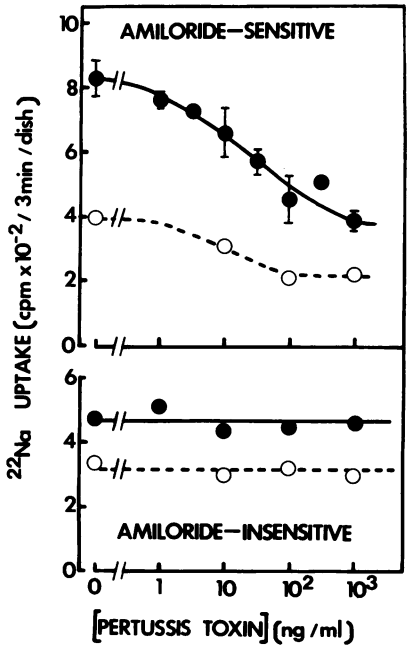

Fig. 4. Concentration-dependent inhibition by pertussis toxin of α-thrombin-stimulated amiloride-sensitive $^{22}Na^+$ uptake. Initial rates of $^{22}Na^+$ uptake were measured in the presence or absence of the amiloride analog, as described under Materials and methods. Cells were pre-treated with increasing concentrations of pertussis toxin and were incubated with (●) or without (○) 10^{-1} U/ml of α-thrombin in the $^{22}Na^+$-labeled medium for 3 min.

of thrombin concentrations from 10^{-3} to 10 U/ml (Figure 3).

Pertussis toxin inhibits α-thrombin-induced activation of the Na^+/H^+ antiport

We previously reported (Paris and Pouysségur, 1984), that the amiloride-sensitive $^{22}Na^+$ influx and its stimulation by α-thrombin could be readily determined by shifting CCL39 cells to a low extracellular Na^+ concentration such as 1 mM. The moderate cytoplasmic acidification thus resulting from Na^+ gradient inversion appeared to stimulate the basal rate of the resting Na^+/H^+ antiporter, allowing a more accurate measurement of the growth factor-induced activation. We applied this method to cells treated with increasing concentrations of pertussis toxin. Initial rates of both amiloride-sensitive and insensitive $^{22}Na^+$ influx were measured with and without 10^{-1} U/ml of α-thrombin. As shown in Figure 4 (lower panel), pertussis toxin treatment did not affect the amiloride-insensitive flux, nor its stimulation by thrombin. The exact nature of this flux is so far unknown. In contrast, a concentration-dependent inhibition by pertussis toxin was observed in the amiloride-sensitive $^{22}Na^+$ uptake, mediated by the Na^+/H^+ antiporter (Figure 4, upper panel). In the presence of 10^{-1} U/ml of α-thrombin, the amiloride-sensitive $^{22}Na^+$ influx was maximally inhibited by ~50%, with half-maximal effect at 20 ng/ml of toxin. A significant decrease, with a similar dose-response, was also observed in unstimulated cells, suggesting that pertussis toxin treatment affects not only the thrombin-induced activation of the antiporter but also its functioning in the resting state.

Under physiological conditions (140 mM Na^+ in the extracellular medium), the thrombin-induced activation of the Na^+/H^+ antiporter results in a cytoplasmic alkalinization of CCL39 cells (L'Allemain *et al.*, 1984b). To define further the inhibitory effect of pertussis toxin on the thrombin-stimulated Na^+/H^+ exchange, we therefore examined how the increase in intracellular pH (pH_i) was affected by toxin treatment. As shown in Figure 5 (left), the thrombin-induced increase in pH_i, measured after 5 min, was markedly reduced in pertussis toxin-treated cells. At 10^{-1} U/ml of α-thrombin, the cytoplasmic alkalinization measured in cells pre-treated with 1 μg/ml of toxin was only half of the control value. Half-maximal effect was produced by ~70 ng/ml of pertussis toxin. It must be noted that pertussis toxin *per se* has no effect on pH_i in G_0-arrested CCL39 cells, despite its inhibitory effect on the amiloride-sensitive $^{22}Na^+$ flux (Figure 4). The reason is probably that, unless stimulated by an acid load, the Na^+/H^+ exchange is extremely slow in quiescent cells (Paris and Pouysségur, 1984). Accordingly, its contribution to maintaining the steady-state pH_i must be very minor, since similar pH_i values were measured in G_0-arrested CCL39 cells and its mutant derivative PS120, lacking Na^+/H^+ antiport activity (L'Allemain *et al.*, 1984b).

As for the inhibition of thrombin-stimulated phosphoinositide breakdown, the pertussis toxin effect was more pronounced at low thrombin concentrations. At 10^{-2} U/ml of α-thrombin, the pH_i increase after 5 min was lowered by ~80% after treatment with 1 μg/ml of pertussis toxin (Figure 5, right).

Pertussis toxin has no effect on TPA- or EGF-induced activation of the Na^+/H^+ antiport

Addition of the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA) or of epidermal growth factor (EGF) to G_0-arrested CCL39 cells also induces a rise in pH_i, although these two compounds are very weak mitogens for these cells. This alkalinization results, in both cases, from activation of the Na^+/H^+ antiport since it is abolished by amiloride in CCL39 cells and does not

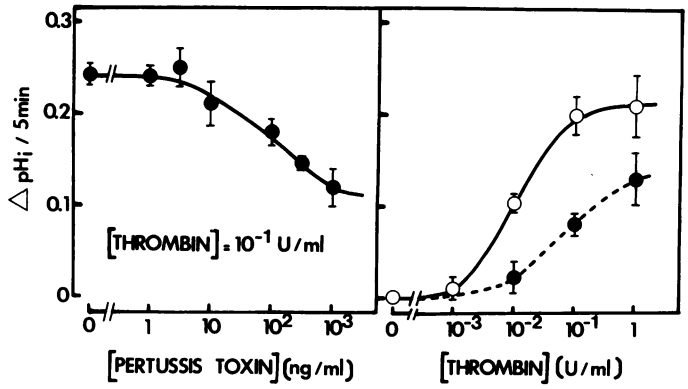

Fig. 5. Inhibition by pertussis toxin of α-thrombin-induced cytoplasmic alkalinization. Dose-response curves for pertussis toxin **(left)** and α-thrombin **(right)**. Increase in intracellular pH (pH_i) was measured with [^{14}C]benzoic acid 5 min after thrombin addition. **Left:** cells were pre-treated with increasing concentrations of pertussis toxin and stimulated with 10^{-1} U/ml of α-thrombin. **Right:** cells were pre-treated with (●) or without (○) 1 μg/ml of pertussis toxin and stimulated with increasing concentrations of α-thrombin. Intracellular pH in unstimulated cells averaged 7.18 ± 0.03, with or without toxin treatment.

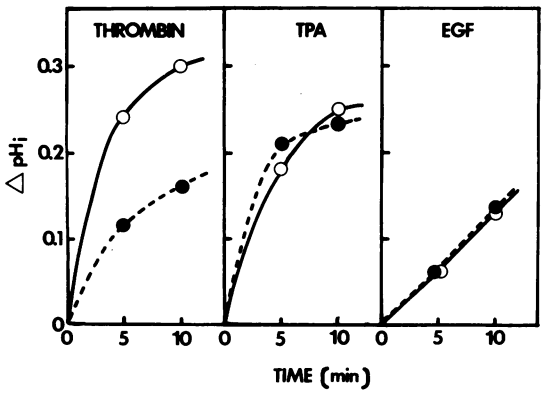

Fig. 6. Comparative effect of pertussis toxin on α-thrombin, TPA- or EGF-induced cytoplasmic alkalinization. G_0-arrested CCL39 cells were pre-treated with (●) or without (●) 1 μg/ml of pertussis toxin and stimulated with 10^{-1} U/ml of α-thrombin **(left)**, 100 ng/ml of TPA **(middle)** or 100 ng/ml of EGF **(right)**. The increase in intracellular pH was determined after 5 and 10 min, with parallel unstimulated cultures.

occur in the Na^+/H^+ antiport-deficient mutant PS120 (data not shown). Interestingly, pre-treatment of CCL39 cells with 1 μg/ml of pertussis toxin does not affect the time-course of pH_i increase after stimulation by TPA or EGF, in sharp contrast with the inhibition of thrombin-induced alkalinization (Figure 6).

Discussion

The present studies demonstrate that prior treatment of G_0-arrested hamster fibroblasts with pertussis toxin results in marked inhibition of two early responses to the mitogen α-thrombin:

(i) generation of inositol phosphates, in particular IP_3 and IP_2, reflecting the activation of a PIP_2-specific phospholipase C, and (ii) activation of the Na^+/H^+ antiporter, measured either by the amiloride-sensitive $^{22}Na^+$ influx or by the increase in pH_i. Since the only biochemical effect reported so far for this bacterial toxin is its capacity to modify a family of GTP-binding proteins by ADP-ribosylation (Ui, 1984; Gilman, 1984), our data strongly suggest that such a regulatory G protein might mediate the thrombin-induced activation of the PIP_2-specific phospolipase C.

The best characterized substrate of pertussis toxin is the inhibitory guanine nucleotide-binding protein N_i (or G_i) of the hormone-sensitive adenylate cyclase system (Ui, 1984; Gilman, 1984; Jakobs *et al.*, 1984). Interestingly, thrombin has been shown to inhibit adenylate cyclase via N_i in platelet membranes (Aktories and Jacobs, 1984), in 3T3 fibroblasts (Murayama and Ui, 1985), as well as in CCL39 cells (Limbird, personal communication). In the three systems, pertussis toxin inhibits this effect of thrombin. Thus, these observations raise the questions of whether N_i could be the signal transducer involved in thrombin-induced activation of phosphatidylinositol turnover and, if so, whether this action is due to a decrease in cellular cAMP. Such a role for N_i in phospholipase C activation has indeed been proposed recently in mast cells (Nakamura and Ui, 1985) and in neutrophils (Volpi *et al.*, 1985), but no direct link between N_i and phospholipase C has been clearly established yet.

In any case, adenylate cyclase does not seem to be involved in this activation pathway, as indicated by the following observations. First, no significant change in the basal level of intracellular cAMP could be detected in G_0-arrested CCL39 cells upon addition of α-thrombin nor after pertussis toxin treatment (not shown). Moreover, if phospholipase C were activated by a decrease in cAMP, then the thrombin-induced stimulation of phosphoinositide hydrolysis and Na^+/H^+ exchange should be suppressed by a massive supply of exogenous cAMP. Actually, incubation of CCL39 cells for 30 min with varying concentrations of 8-BrcAMP (up to 1 mM) did not impede the subsequent increase in pH_i resulting from thrombin addition (data not shown).

It must be stressed that N_i is not the only substrate known for pertussis toxin and that some other member of the increasing family of G proteins might well be responsible for phospholipase C activation, such as the 39-kd G_0 protein, initially isolated from brain (Sternweis and Robishaw, 1984; Neer *et al.*, 1984), but apparently also present in other tissues (Malbon *et al.*, 1984, 1985), or the *ras* gene product (Berridge and Irvine, 1984). Indeed, although sensitivity of the *ras* proteins to pertussis toxin remains to be demonstrated, striking sequence homologies have been found between these proteins and transducin, a GTP-binding protein of retinal rod cells, known to be ADP-ribosylated by pertussis toxin (Medynski *et al.*, 1985; Yatsunami and Khorana, 1985).

The second interesting observation in this study is the parallelism between inhibition of α-thrombin-induced phosphoinositide hydrolysis and Na^+/H^+ antiport activation, suggesting a causal relationship between the two events. Indeed, this is consistent with the stimulatory effect of TPA on the Na^+/H^+ antiport, since TPA is known to directly activate the protein kinase C, in place of diacylglycerol generated by phosphoinositide hydrolysis (Nishizuka, 1984). However, pertussis toxin might have exerted a direct inhibition on the antiporter. It was therefore of interest to rule out this possibility by examining the effect of pertussis toxin on other antiporter activators, the action of which is not mediated by phospholipase C. The results presented here (Figure 6) clearly show that pertussis toxin is without effect on TPA- or EGF-induced activation of Na^+/H^+ exchange. How EGF stimulates the antiporter is not known, but this pathway appears to be independent of the phosphoinositide cycle, since we failed to detect any inositol phosphate production upon EGF addition, even in hamster fibroblasts mitogenically responsive to this growth factor (L'Allemain *et al.*, in preparation). In 3T3 cells also EGF-induced stimulation of the antiporter has been shown recently to be independent of protein kinase C (Vara and Rozengurt, 1985).

In contrast to EGF, serum was found to stimulate the PI turnover in CCL39 cells (L'Allemain *et al.*, in preparation) and, strikingly, the serum-induced increase in pH_i was also markedly inhibited by pertussis toxin treatment (data not shown). Since it has been shown using the specific thrombin inhibitor hirudin (Van Obberghen-Schilling *et al.*, 1982), that thrombin does not contribute to mitogenicity of serum, our data altogether indicate that (i) pertussis toxin effects are not restricted to thrombin, but rather extend probably to all PI turnover-stimulating mitogens; (ii) pertussis toxin does not impair *per se* the activation mechanism of the Na^+/H^+ antiporter, but reduces this activation as a consequence of inhibition of the PI response.

Therefore, it can be concluded that, at least in part, the thrombin-induced stimulation of Na^+/H^+ exchange is mediated by some second messenger(s) arising from the PI cycle. Diacylglycerol is likely to be one of these, considering the stimulatory effect of TPA. However, protein kinase C does not seem to be responsible for the totality of the thrombin effect on Na^+/H^+ antiport, as suggested by the following observations (unpublished data). First, thrombin induces a further alkalinization in CCL39 cells maximally stimulated with TPA. Second, in contrast to thrombin, TPA does not stimulate to a detectable extent the amiloride-sensitive $^{22}Na^+$ influx under our standard assay conditions, suggesting that, in CCL39 cells, the antiport is activated to a lower extent by phorbol esters than by thrombin. As discussed by Cassel *et al.* (1985), pH_i increase may be in some cases a more sensitive measure of an activated Na^+/H^+ exchange than the amiloride-sensitive Na^+ influx.

If the pathways other than protein kinase C involved in Na^+/H^+ activation remain to be elucidated, their existence may explain the apparent shift in sensitivity to pertussis toxin between phospholipase C (Figure 2) and amiloride-sensitive $^{22}Na^+$ influx (Figure 4). It must also be noted that, whereas the sensitivity to pertussis toxin of inositol phosphate formation was determined at 10^{-2} U/ml of α-thrombin, the growth factor concentration was raised to 10^{-1} U/ml for measurement of Na^+/H^+ exchange, to allow a better stimulation and a more accurate determination of the inhibition. Since maximal inhibition by pertussis toxin decreases at higher thrombin concentrations (Figure 3), a shift in the dose-dependence curve for pertussis toxin with increasing thrombin concentrations cannot be excluded.

The increase in pH_i, measured 5 min after thrombin addition, appears even less sensitive to pertussis toxin than the amiloride-sensitive $^{22}Na^+$ influx. However, only the initial rates of pH_i elevation are expected to closely reflect the Na^+/H^+ exchange activity; since alkalinization induced by 10^{-1} U/ml of α-thrombin in control cells was not rigorously linear over 5 min (Figure 6), inhibition by low concentrations of pertussis toxin may have been underestimated.

It is noteworthy that pertussis toxin affects essentially the rate of pH_i rise, rather than the final steady-state pH_i value. After 30 min incubation with 10^{-1} U/ml of α-thrombin, pH_i was only slightly lower in toxin-treated cells than in control cells (not shown).

In summary, the present report provides some evidence for the involvement of a GTP-binding protein in thrombin-induced activation of a PIP_2-specific phospholipase C, and for a causal relationship between phosphoinositide hydrolysis and activation of the Na^+/H^+ antiport. But obviously, further experimental work is required to demonstrate more directly the role of GTP in the signal transduction and to better elucidate the different pathways leading to Na^+/H^+ antiport activation.

Materials and methods

Cell culture

The Chinese hamster fibroblast line CCL39 (American Type Culture Collection) was cultivated in Dulbecco's modified Eagle's medium (DME; Gibco) containing 5% fetal calf serum, penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37°C in 5% CO_2/95% air.

Confluent monolayers in 35-mm dishes were rendered quiescent by a 20-h incubation in a serum-free medium (DME or 1:1 ratio of DME:Ham's F12, as indicated below).

Measurement of phosphoinositide breakdown

Confluent cultures were arrested and labeled to equilibrium with [^{3}H]inositol (2 μCi/ml) for 20 h in serum-free DME medium. Pertussis toxin was added directly to this medium for an additional 3 h incubation. Then pertussis toxin-treated (or control) cells were extensively washed with a Hepes-buffered solution containing 20 mM LiCl (110 mM NaCl, 50 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM Hepes/NaOH pH 7.4) and incubated for 15 min in this solution. Thereafter, the cultures were stimulated with α-thrombin, at the indicated concentrations, in 1 ml of fresh Li^+-containing solution. The reaction was stopped by removing the medium and immediately extracting the cells with 0.5 ml of 10% (w/v) $HClO_4$. Alternatively, when [^{3}H]inositol was maintained in the extracellular medium during thrombin stimulation, the cultures were extensively washed with ice-cold saline solution before extraction with perchloric acid. Separation of [^{3}H]inositol phosphates was carried out essentially as described by Bone *et al.* (1984): the acid extract was neutralised with KOH after addition of 50 mM Mops buffer. The $KClO_4$ precipitate was eliminated by centrifugation and the supernatant applied to a 0.6 ml column of Dowex 1 × 8 (formate). Free inositol, glycerophosphoinositol and inositol mono-, bis- and trisphosphates were successively eluted with 2 × 2 ml of (i) H_2O, (ii) 60 mM ammonium formate/5 mM sodium tetraborate, (iii) 180 mM ammonium formate/5 mM sodium tetraborate, (iv) 0.5 M ammonium formate/0.1 M formic acid, (v) 1.5 M ammonium formate/0.1 M formic acid, respectively. Alternatively, total inositol phosphates were eluted in a single step with 5 ml of 1.5 M ammonium formate/0.1 M formic acid. Radioactivity in the eluates was determined by scintillation counting.

Measurement of intracellular pH and $^{22}Na^+$ uptake

Confluent cultures were arrested by a 20-h incubation in serum-free DME/Ham's F12 medium (1:1). Then, the cells were incubated with or without pertussis toxin for 3 h in DME medium buffered at pH 7.4 with 20 mM Hepes (in the absence of CO_2). After this treatment, cells were washed with a balanced salt solution consisting of 130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM Hepes/NaOH pH 7.4 and equilibrated in this solution for 15 min. To measure the growth factor-induced cytoplasmic alkalinization, the cultures were stimulated with α-thrombin, TPA or EGF in the same saline solution containing 1 μCi/ml of [^{14}C]benzoic acid. Intracellular pH was calculated from the equilibrium distribution of this weak acid, as previously described (L'Allemain *et al.*, 1984b). Initial rates of $^{22}Na^+$ uptake were determined in a modified salt solution (K^+-free, 1 mM Na^+, isotonicity maintained with choline chloride), in the presence of 1 mM ouabain, as previously described (Paris and Pouysségur, 1984). The amiloride-insensitive flux was measured in the presence of 50 μM of the potent amiloride analog 5-N-propenyl N-methyl-amiloride (L'Allemain *et al.*, 1984a).

Materials

Pertussis toxin was purchased from List Biological Laboratories (Campbell, CA). Highly purified human α-thrombin (2660 NIH units/mg) was generously provided by Dr J.W.Fenton II (New York State Department of Health, Albany, NY). TPA was obtained from Sigma. The amiloride analog was a gift of Dr E.Cragoe, Jr. (Merck Sharp and Dohme Research Laboratory).

Myo-[2-^{3}H(N)]inositol and [7-^{14}C]benzoic acid were from New England Nuclear, and ^{22}NaCl from the Radiochemical Centre (Amersham). Growth factors (α-thrombin, TPA or EGF) were added to the cultures from 50-fold concentrated solutions in 2 mg/ml of bovine serum albumin (BSA). The same concentration of BSA was added to control cells.

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