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Mechanism of Enhanced Sensitivity to Bradykinin in Pertussis Toxin-Treated Fibroblasts: Toxin Increases Bradykinin-Stimulated Prostaglandin Formation

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SUMMARY

Exposure of animals to pertussis toxin results in increased sensitivity to agents such as bradykinin. To elucidate the molecular mechanisms underlying the effects of toxin, bradykinin responsiveness was examined in control and intoxicated human fibroblasts. Exposure of fibroblasts to toxin resulted in a loss of inhibitory agonist action on adenylate cyclase, elevation of basal cAMP, and ADP-ribosylation of a 41-kDa protein, which was identified as $G_{i\alpha}$, a component of adenylate cyclase, by its pattern of immuno-cross-reactivity with a family of antibodies to guanyl nucleotide-binding proteins, which are pertussis toxin substrates, and by the presence of an mRNA species with characteristics of a form of $G_{i\alpha}$. Bradykinin increased prostaglandin accumulation to a greater extent in toxin-treated than in control fibroblasts. Agents such as cholera toxin, which elevated cAMP, also increased bradykinin-induced prostaglandin production. These data are consistent with the hypothesis that the enhanced sensitivity to bradykinin after pertussis toxin treatment results from modification of $G_{i\alpha}$ and increased cAMP, leading to enhanced formation of prostaglandins in response to bradykinin.

Intracellular cyclic AMP is critical to the regulation of many metabolic pathways. In human fibroblasts, formation of cAMP is controlled by a hormone-sensitive adenylate cyclase system that is under the dual regulation of stimulatory and inhibitory agents (1–4). cAMP content is increased by prostaglandins such as PGI_2 and PGE_2, choleragen, and β-adrenergic agonists such as isoproterenol (1–3); adenylate cyclase is inhibited by muscarinic cholinergic agonists such as carbachol (4). These agonists increase or suppress adenylate cyclase activity through stimulatory or inhibitory receptors coupled through different GTP-binding proteins termed G_s and G_i, respectively, to a catalytic unit responsible for the conversion of ATP to cAMP (5).

Ui and co-workers and other laboratories demonstrated that pertussis toxin (islet-activating protein), a secretory product of *Bordetella pertussis*, can block the action of inhibitory ligands on adenylate cyclase by catalyzing ADP-ribosylation of a 41-kDa component of G_i (6–12). ADP-ribosylated G_i exhibits reduced sensitivity to guanyl nucleotides and an inability to transduce effects of inhibitory agonist-receptor interaction to the catalytic unit of adenylate cyclase (6–8, 13, 14). Pertussis toxin-catalyzed ADP-ribosylation of G_i also alters the effects of stimulatory agonists on cyclase. In some cells, exposure of cells to pertussis toxin enhances basal cAMP and responses to stimulatory agonists, even in the absence of added inhibitory ligands (7–9, 15–18). It was hypothesized that in these cells cyclase was under basal inhibitory tone by G_i that was relieved after ADP-ribosylation of the 41-kDa protein by pertussis toxin (7–9, 15–18).

Bradykinin, a nonapeptide, is involved in many biological processes (19, 20). For example, it is believed to be a mediator of the inflammatory response and is a potent vasodilator (19, 20). The effects of bradykinin on its target tissues result in part from generation of arachidonate metabolites and formation of

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ABBREVIATIONS: PGI_2, prostaglandin I_2; G_s, the stimulatory GTP-binding protein of cyclase; G_i, the inhibitory GTP-binding protein of cyclase; HBSS, Hanks' balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; PGE_2, prostaglandin E_2; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; $G_{o\alpha}$, α subunit of a GTP-binding protein, $G_{o\alpha\beta\gamma}$, purified from bovine brain; $G_{i\alpha}$, a subunit of the GTP-binding protein, $G_{i\alpha\beta\gamma}$; multiple G_i-like proteins exist, which G_i regulate(s) adenylate cyclase or bradykinin response is unknown.

TABLE 1

Effect of pertussis toxin on carbamylcholine-induced decrease in cellular cAMP content

Fibroblasts were incubated with pertussis toxin (1.35 μg/culture dish) for 24 hr and then assayed for 3 min at 37° as described in Methods in the presence or absence of 100 μM carbamylcholine. The experiment was repeated seven times. Effect of carbamylcholine on basal cAMP in the absence of pertussis toxin was statistically significant ($p < 0.001$). After exposure to pertussis toxin, carbamylcholine did not affect the cAMP content of fibroblasts ($p = 0.4$). Treatment with pertussis toxin, however, caused a significant elevation in cAMP ($p < 0.01$).

Carbamylcholine	cAMP content	
	No toxin	Plus toxin
	pmol/mg	
None	100[a]	158 ± 52.2
100 μM	56.4 ± 21.9	166 ± 38.2

[a] Average basal cAMP was set at 100 for each experiment. All data are given relative to this figure. The data are averaged from all seven experiments.

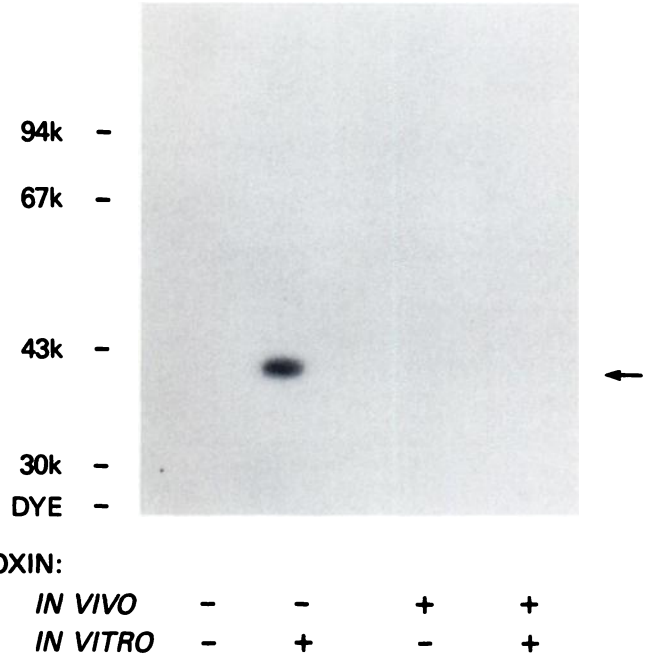

Fig. 1. [^{32}P]ADP-ribosylation by pertussis toxin of a 41-kDa protein in membranes from human fibroblasts. Human fibroblasts were incubated with or without pertussis toxin (1.72 μg) for 24 hr. The cells were washed as described in Methods, scraped in 1.5 ml of Dulbecco's phosphate-buffered saline, and homogenized with 30 strokes in a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 12,000 × *g* for 30 min; the particulate fraction was suspended in 10 mM Tris (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, and 1 mM NaN_3. Toxin-catalyzed [^{32}P]ADP-ribosylation of membrane particulate fraction (186 μg of protein) was carried out in a total volume of 400 μl containing 15 mM thymidine, 10 μM [^{32}P]NAD (3 μCi), 0.75 mM ATP, 200 μM guanyl-5-yl-β-γ-imidodiphosphate, 37.5 mM sodium phosphate buffer (pH 7.5), and 4 μg of activated pertussis toxin in 7.5 mM glycine (pH 8.0), and 3 mM dithiothreitol (37). Samples were incubated for 30 min at 30°, and the reaction was terminated by the addition of 1.5 ml of 6.25% trichloroacetic acid. The proteins were electrophoresed in 12% SDS-polyacrylamide gels (50 μg of protein/sample well); ADP-ribosylation was quantified by autoradiography (38).

the cyclic nucleotides, cAMP and cGMP (21–24). Human skin fibroblasts grown in culture are responsive to bradykinin (bradykinin ≫ des-arg-bradykinin) (25). Interaction of bradykinin with its receptor leads to phospholipase activation, arachidonate release, and formation of PGI_2 and PGE_2 (23, 25, 26). Because these prostaglandins increase fibroblast cAMP content, it has been hypothesized that the stimulation of cAMP by bradykinin results from the generation of PGI_2 and PGE_2 (23, 25, 26). Injection of pertussis toxin into experimental animals leads to enhanced sensitivity to autocoids such as histamine and bradykinin (27). We have used cultured human fibroblasts as a model system in which to study possible mechanisms for increased responsiveness to bradykinin after toxin treatment.

Experimental Procedures

Materials

Fetal calf serum was purchased from KC Biologicals (Lenexa, KS); cholera toxin from Schwarz/Mann (Orangeburg, NJ); Freon from E. I. Dupont de Nemours (Wilmington, DE); trioctylamine, 8-bromo-cAMP, and isoproterenol from Sigma Chemical Co. (St. Louis, MO); glutamine and HBSS from GIBCO (Grand Island, NY); forskolin from Calbiochem (La Jolla, CA); minimal essential medium with Earle's salts, bicarbonate, and nonessential amino acids from the National Institutes of Health media unit; tissue culture flasks and dishes from Falcon (Lincoln Park, NJ); 6-keto-$PGF_{1\alpha}$, PGE_2, and PGE_1 from Seragen (Boston, MA); bradykinin from Beckman (distributor for Peptide Institute, Protein Research Foundation, Osaka, Japan); [^{3}H]6-keto-$PGF_{1\alpha}$ (130 Ci/mmol), PGE_2, and cAMP radioimmunoassay kits from New England Nuclear (Boston, MA).

Methods

Toxin preparation and incubation. Pertussis toxin was purified as described previously (28) and was added to fibroblasts 24 hr before experiments or as indicated.

Cell culture. Fibroblasts were grown in Eagle's minimal essential medium with 10% fetal calf serum as described earlier (25). Fourteen days before experiment, 60 × 15 mm dishes were seeded with 250,000 cells; medium was changed on day 12. In all instances, fibroblasts were confluent before the experiment was initiated. Pertussis toxin did not appear to alter cell morphology or adherence to culture dishes.

Incubations with pertussis toxin, 8-bromo cAMP, choleragen, forskolin, and IBMX. Twenty-four hours before experiments or as indicated, the agent was added to complete medium containing fetal calf serum. The final concentrations were as follows: choleragen, 1 μg/ml; 8-bromo cAMP, 0.5 mM; forskolin, 10 μM; and IBMX, 67 μM. All incubations were performed in triplicate; data are expressed as mean ± standard error. Experiments were performed at least twice with similar results.

Incubation with bradykinin, pGE_1, and isoproterenol. On the day of experiment, growth medium was removed and the cells were washed with HBSS (3 ml). After the cells had incubated for 10 min at 37° with 2 ml of HBSS, the medium was replaced with 2 ml of HBSS with the indicated agonist. After 3 min, medium was collected for prostaglandin determination. Cold 5% trichloroacetic acid (1.5 ml) was added to the cell layer; the cells on the dish were frozen at −70° for 1 hr and thawed, and 1 ml was removed for cAMP determination. All incubations were performed in triplicate; data are expressed as mean ± standard error. Experiments were performed at least twice with similar results.

6-Keto-$PGF_{1\alpha}$ and PGE_2 determinations. PGE_2 was determined with a radioimmunoassay kit from New England Nuclear. PGI_2 was assayed as its hydrolysis product 6-keto-$PGF_{1\alpha}$ by radioimmunoassay using [^{3}H]6-keto-$PGF_{1\alpha}$ from New England Nuclear and antibody from Seragen by the method given by Seragen. All assays were performed in duplicate.

cAMP determination. The dishes containing the trichloroacetic acid-treated cell layers were thawed and 1 ml was removed for extraction with trioctylamine in Freon (29). cAMP was determined by radioimmunoassay. All assays were performed in duplicate.

Protein. Cells were solubilized in 1 N NaOH; a sample was assayed

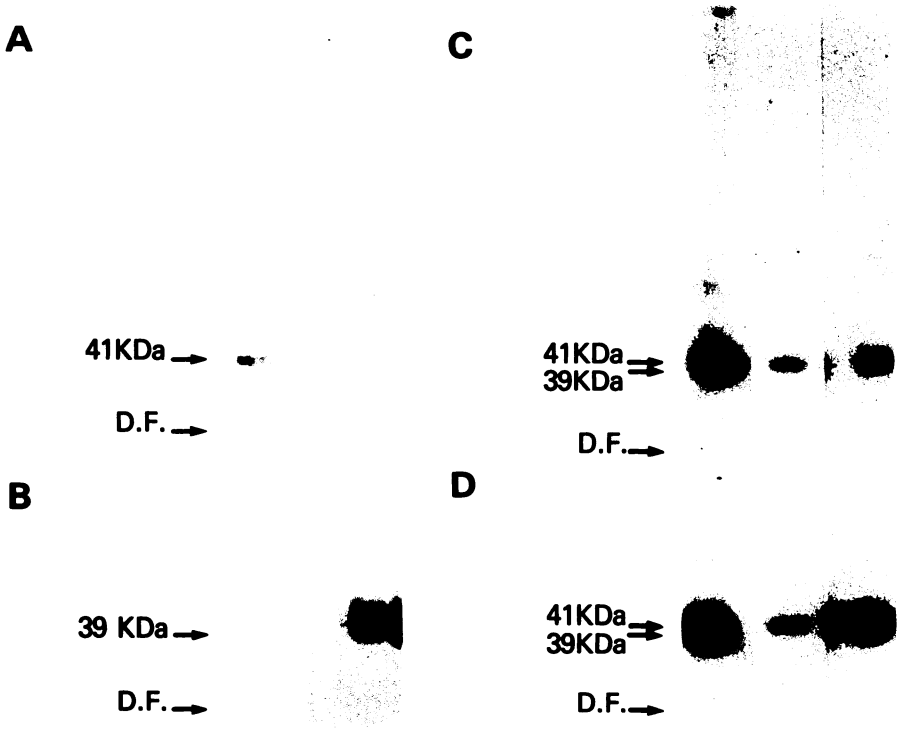

Fig. 2. Immunochemical characterization of the pertussis toxin substrate in human fibroblasts. Confluent fibroblasts were washed, harvested, and homogenized with 250 mM sucrose, 20 mM Tris, pH 7.5, 5 mM $MgCl_2$. The homogenate was centrifuged at 12,000 × *g* for 40 min at 4°; the particulate fraction was suspended in 10 mM Tris, pH 8.0, 1 mM EDTA, 1 mM azide, and 1 mM dithiothreitol. Fibroblast particulate fractions (~50 μg), $G_{i\alpha\beta\gamma}$ containing 0.3 μg of $G_{i\alpha}$ subunit, or 3 μg of $G_{o\alpha}$ subunit were ADP-ribosylated with 1 μg of pertussis toxin (List Biological Laboratories, Campbell, CA) and 10 μM [^{32}P]NAD (2 μCi) at 30° for 1 hr as described by Burns *et al.* (37). The reaction was stopped by addition of cold 7.5% trichloroacetic acid; 5 μg of bovine serum albumin was added to each sample. The precipitated proteins were electrophoresed on two 12% SDS-polyacrylamide gels according to the method of Laemmli (38), and the gels were transferred to nitrocellulose paper ("Western blots") (39). One nitrocellulose blot was incubated first with murine monoclonal anti-T_α-antibody and then with goat anti-mouse IgG-horseradish peroxidase conjugate (Bio-Rad, Richmond, CA) to identify $G_{i\alpha}$. The blot was then exposed to Kodak X-Omat AR film. The second blot was incubated with rabbit serum against $G_{o\alpha}$ and then goat anti-rabbit IgG-horseradish peroxidase to identify $G_{o\alpha}$. This blot was also exposed to Kodak X-Omat AR film. A, the nitrocellulose blot immunoreacted with monoclonal anti-T_α antibodies; B, the nitrocellulose blot immunoreacted with antiserum against $G_{o\alpha}$; C and D, are autoradiograms of the two nitrocellulose blots of the SDS-polyacrylamide gels used in A and B, respectively. *Lane 1*, $G_{i\alpha\beta\gamma}$ (0.3 μg of $G_{i\alpha}$); *lane 2*, human fibroblast membrane (50 μg); *lane 3*, $G_{o\alpha}$ (3.0 μg). *D.F.*, dye front. *41 kDa*, the subunit of $G_{i\alpha}$; *39 kDa*, of $G_{o\alpha}$.

by the method of Lowry *et al.* (30) using bovine serum albumin as a standard.

Preparation of monoclonal antibody. Monoclonal antibody 6E4 (31) was derived from a fusion of the myeloma cell line Sp2/0-Ag14 with spleen cells from a BALB/c mouse immunized with purified transducin. It reacted strongly with T_α, $G_{i\alpha}$, and, to a lesser extent, with $G_{o\alpha}$ (<3%). For Western blots, nitrocellulose sheets were incubated with 6E4 culture supernatant (~10.0 μg/ml).

Purification of T_α subunit, $G_{o\alpha}$ subunit, and $G_{i\alpha\beta\gamma}$ complex. Transducin ($T_{\alpha\beta\gamma}$) was purified from fresh bovine retina by the method of Kühn (32). T_α was separated from $T_{\beta\gamma}$ using Blue Sepharose CL-6B (33).

$G_{i\alpha\beta\gamma}$ complex was purified from rabbit liver membranes in the absence of $AlCl_3$, NaF, and $MgCl_2$ by the method of Sternweis *et al.* (34) with minor modifications. $G_{i\alpha\beta\gamma}$ complex was separated from $G_{s\alpha\beta\gamma}$ complex using a heptylamine Sepharose column with a gradient (8 times column volume) of 0.25% cholate and 200 mM sodium chloride in 20 mM Tris (Cl^-), pH 8.0, 1 mM EDTA, 2 mM dithiothreitol, 0.25 M sucrose, and 1 mM azide (buffer A) to 1.3% cholate and 50 mM sodium chloride in buffer A. Fractions containing $G_{i\alpha\beta\gamma}$ were identified by electrophoresis on 12% SDS-polyacrylamide gels, followed by staining with Coomassie Blue. $G_{o\alpha}$ subunit (>96% purity) was purified by a modification of the method of Sternweis and Robishaw (34, 35).

Preparation of antiserum against $G_{o\alpha}$. Preparation and characterization of rabbit antiserum against $G_{o\alpha}$ has been described (36). This antiserum exhibited minimal cross-reactivity with $G_{i\alpha}$.

Results

Incubation of human fibroblasts with pertussis toxin resulted in an increase in basal cAMP content and a loss of responsiveness to inhibitory ligands such as carbamylcholine (Table 1). When membranes were incubated with toxin and [^{32}P]NAD, solubilized, and electrophoresed on SDS-polyacrylamide gels, a 41-kDa band was present in autoradiograms from control, but not toxin-treated, fibroblasts (Fig. 1).

Both $G_{i\alpha}$ and $G_{o\alpha}$ exhibit similar mobilities in SDS-polyacrylamide gel electrophoresis (34). With membranes from human fibroblasts, however, the 41-kDa band on SDS-polyacrylamide gels reacted with monoclonal antibody 6E4 (which cross-reacts with $G_{i\alpha}$) but not with the antiserum against $G_{o\alpha}$ (Fig. 2, A and B). ADP-ribosylation did not affect the ability of the G protein to react with antibody (Fig. 2, C and D). The antibody reactivity of the membrane-bound G proteins was a much more sensitive means to distinguish between them than was their mobility on SDS-polyacrylamide gels.

To determine further the presence of a $G_{i\alpha}$ in fibroblasts, a Northern blot analysis of poly(A)$^+$ RNA was performed with oligonucleotide probes specific for $G_{i\alpha}$ and a cDNA probe for $G_{o\alpha}$ (40). Sequences of the synthetic $G_{i\alpha}$ oligonucleotide probes were determined by comparing the nucleotide sequences for all G proteins and selecting a 24-base probe unique for a form of $G_{i\alpha}$. The oligonucleotide probes hybridized with a 2.2 kb mRNA species consistent with that of $G_{i\alpha 2}$ (Fig. 3) whereas the $G_{o\alpha}$ cDNA did not hybridize with any RNA (data not shown).

Treatment of fibroblasts with pertussis toxin for 18–24 hr slightly increased basal cAMP content and PGI_2 and PGE_2 accumulation and enhanced the response to bradykinin (Table

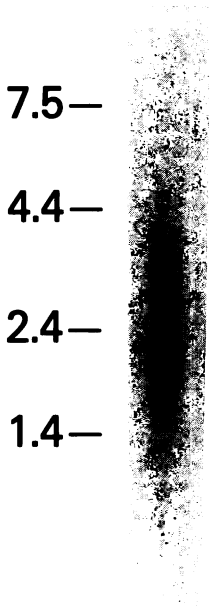

Fig. 3. Identification of $G_{i\alpha 2}$ poly(A)$^+$ RNA in human fibroblasts. Poly(A)$^+$ RNA was isolated from human fibroblasts using the method of Chirgwin (41) followed by oligo(dT) chromatography. The RNA (5 μg) was separated on a formaldehyde/1% agarose gel and transferred to nitrocellulose. The blots were hybridized with a ^{32}P-5′-end-labeled 24-base oligonucleotide probe complementary to sequences reported for the α-subunit of G_i of rat brain [5′-CAGGTCTTCCGGAAGCATGCCTTG-3′; Itoh *et al.* (42)] or mouse macrophages [5′-CAGGTCTTCAGGAAGCAT CCCTTG-3′; Sullivan *et al.* (43)] at low stringency [5× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate)/5× Denhardt's solution (1× Denhardt's = 0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone)/20 mM Tris·HCl, pH 7.5/10% dextran sulfate/100 μg of denatured salmon sperm DNA per ml at 42°]. Filters were washed in 5× SSC/0.5% SDS at 42° for 45 min and 2× SSC/0.5% SDS at 42° for 30 min. The blot hybridized with the rat $G_{i\alpha}$ probe is shown. The *numbers* to the left of the figure represent the positions of the RNA markers in nucleotides (10^{-3}).

2) as well. The time course of the effects of toxin treatment on bradykinin responses is shown in Fig. 4. After 18–24 hr with toxin, effects of bradykinin on cAMP content (Fig. 4A) and PGI_2 and PGE_2 accumulation (Fig. 4B) are enhanced. After treatment with toxin for 48 hr, however, incubation with bradykinin resulted in lower intracellular cAMP content (Fig. 4A) and reduced PGI_2 and PGE_2 (Fig. 4B) formation, when compared with the 24-hr response.

Optimal potentiation of bradykinin stimulation of intracellular cAMP accumulation by a 24-hr incubation with toxin was observed at subsaturating (0.01 μg/ml) rather than saturating (0.2 μg/ml) concentrations of bradykinin (Fig. 5A). The effects of intoxication on bradykinin-stimulated PGI_2 levels were observed, however, at concentrations of bradykinin that produced both suboptimal and maximal effects on intracellular cAMP (Fig. 5B). These observations are compatible with saturation of the receptors responsible for cAMP generation by prostaglandins generated in response to lower bradykinin concentrations. Because the enhanced cAMP responses to bradykinin could result either from enhanced prostaglandin production or increased sensitivity to prostaglandins, the effects of other stimulatory agonists or cAMP were tested. Intoxication, however, did not increase the responsiveness to PGE_1 or isoproterenol (data not shown). The effects of toxin on bradykinin-stimulated PGI_2 production were mimicked in part by agents

TABLE 2

Effect of pertussis toxin on cAMP content and PGI_2 and PGE_2 accumulation

Fibroblasts were incubated with pertussis toxin (0.5 μg/ml) for 24 hr and then incubated for 3 min in the absence or presence of bradykinin (0.01 μg/ml) and assayed for cAMP content and PGI_2 and PGE_2 accumulation as described in Methods. cAMP, PGI_2, and PGE_2 content are presented as mean ± standard error of 25 or 29 experiments. The effect of pertussis toxin is presented as the per cent increase (mean ± standard error) of paired samples and analyzed according to the *t* test for paired samples.

Addition	No. of Expts.	cAMP content		% Increase due to pertussis toxin
		No toxin	Toxin	
		pmol/mg		
None	29	52.8 ± 6.7	69.6 ± 6.7	34.5 ± 10.1 ($p < 0.002$)
BK[a]	29	351.4 ± 33	557.8 ± 59.2	86.5 ± 28.9 ($p < 0.01$)
Addition	No. of Expt.	PGI_2		% Increase due to pertussis toxin
		No toxin	Toxin	
		ng/mg		
None	29	2.8 ± .6	4.8 ± 1.2	70.2 ± 9.6 ($p < 0.001$)
BK	29	17.6 ± 3.2	34.4 ± 5.6	135.2 ± 31.3 ($p < 0.001$)
Addition	No. of Expts.	PGE_2		% Increase due to pertussis toxin
		No toxin	Toxin	
		ng/mg		
None	25	0.22 ± .02	0.37 ± .05	62.0 ± 10.4 ($p < 0.001$)
BK	25	1.3 ± .25	3.1 ± .56	186.8 ± 61 ($p < 0.01$)

[a] BK, bradykinin.

that elevate intracellular cAMP, such as 8-bromo-cAMP, choleragen, IBMX, and forskolin (Fig. 6).

Discussion

Injection of pertussis toxin into experimental animals results in an enhanced sensitivity to several agents; included among these are histamine, bradykinin, and serotonin (26). At present, the effects of pertussis toxin have all been attributed to ADP-ribosylation of G_i, the inhibitory GTP-binding component of cyclase, or to modification of G_o, a guanyl nucleotide-binding protein that couples to inhibitory receptors but apparently not to the catalytic unit of the adenylate cyclase system (5, 35, 44, 45). ADP-ribosylation of G_i appears to impair its ability to couple to receptors. This covalent modification inactivates G_i, resulting in a loss both of inhibitory agonist suppression of cyclase activity and of high affinity binding of inhibitory agonists to their receptors (13, 14, 45). Exposure of cells in culture to pertussis toxin led to an enhanced responsiveness to stimulatory agonists and an increase in basal cAMP (7–9, 15, 16); these and other data have been interpreted as showing that the cyclase system is under a chronic inhibition in the absence of exogenous inhibitory ligands (7–9, 15–18). Thus, based on these studies, it would appear that, in cyclase systems subject to dual regulation by inhibitory and stimulatory agonists, pertussis toxin-catalyzed ADP-ribosylation can relieve the inhibitory tone and thereby can both promote the activity of stimulatory agonists as well as increase basal cAMP content. In human fibroblasts, however, it appears that although pertussis toxin enhances basal cAMP it does not increase the sensitivity of the cells to stimulatory agonists such as β-adrenergic agents and PGE_1; thus, the mechanisms responsible for enhanced basal cAMP and increased responsiveness to stimulatory agonists may differ.

Fibroblasts are similar to other cultured cells treated with pertussis toxin in that they display an increase in basal cAMP

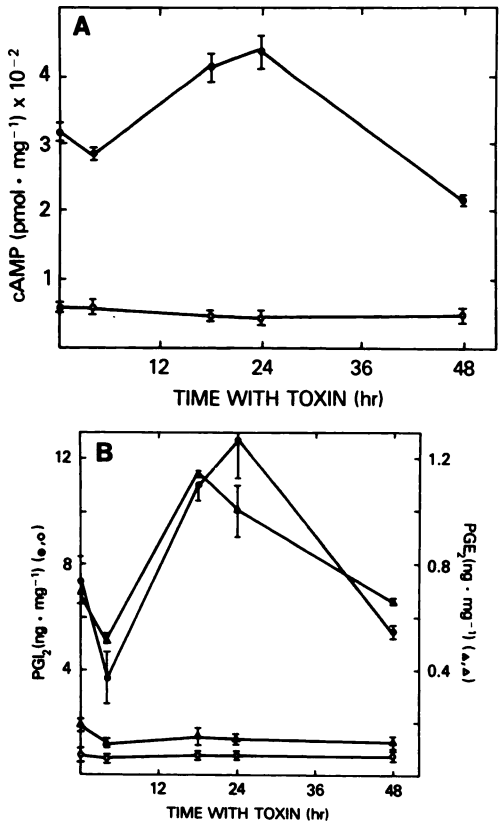

Fig. 4. Effect of pertussis toxin on cAMP accumulation and PGI_2 and PGE_2 formation in the presence of bradykinin. A, Cells were incubated in triplicate with or without pertussis toxin (1.3 μg) for the indicated times, washed, and incubated for 3 min with (●) or without (○) bradykinin, 0.01 μg/ml. cAMP was determined as noted in Experimental Procedures. Data are expressed as mean of triplicate determinations ± standard error. This experiment was repeated twice with similar results. B, Cells were incubated in triplicate with (●, ▲) or without (○, △) bradykinin. PGI_2 (●, ○) and PGE_2 (▲, △) were quantified as described in Experimental Procedures. Data are expressed as mean of triplicate determinations ± standard error. This experiment was repeated twice with similar results.

and a loss of responsiveness to inhibitory agonists, such as the muscarinic cholinergic agent carbamylcholine (Table 1). In contrast to the observations in C6 glioma cells (16) and NG108-15 hybrid cells (13), exposure of human fibroblasts to pertussis toxin did not enhance the response to stimulatory ligands such as β-adrenergic agonists and PGE_1, respectively. Exposure of cells to pertussis toxin did, however, increase the effect of bradykinin on PGI_2 and PGE_2 production. Because these prostaglandins increase fibroblast cAMP content, the increased effect of bradykinin on cAMP accumulation in pertussis toxin-treated cells is presumably indirect, secondary to increased prostaglandin production. In addition to pertussis toxin, chronic exposure of human fibroblasts to other agents that elevate cAMP (e.g., forskolin, IBMX, choleragen, and 8-bromo-cAMP) also resulted in enhanced PGI_2 and PGE_2 production in response to bradykinin. The effect of these agents on cAMP was dependent on time of incubation and the different time courses and intracellular cAMP levels achieved may in part explain the variation in bradykinin-stimulated PGI_2 and PGE_2 levels. In addition, these agents differ in the mechanisms by which they enhance cAMP. 8-Bromo-cAMP bypasses the adenylate cyclase system and presumably directly activates cAMP-dependent protein kinase. Forskolin activates the catalytic unit of adenylate cyclase whereas IBMX inhibits cyclic nucleotide phosphodiesterases, thereby blocking cAMP degradation. Cholera toxin ADP-ribosylates G_s, the stimulatory G protein, and potentiates its activity on adenylate cyclase. The fact that these agents act by different mechanisms to elevate cAMP but have a common effect on bradykinin-stimulated prostaglandin formation is consistent with the hypothesis that cAMP is responsible for the effect. These studies are compatible with the proposal that the effects of pertussis toxin on bradykinin responsiveness result from inactivation of G_i, leading to loss of inhibitory tone on the stimulatory arm of the cyclase and thus an increase in basal cAMP content. cAMP in turn presumably alters those pathways of arachidonate metabolism and prostaglandin production that are regulated by bradykinin. Thus, it would appear not only that, in human fibroblasts, cAMP accumulation is rapidly increased by prostaglandin produced in response to bradykinin but that also prolonged elevations in cAMP serve as a stimulus for further prostaglandin formation.

In other studies, different effects of pertussis toxin on arachidonate metabolism have been reported. In neutrophils and mast cells, ADP-ribosylation appears to result in an inhibition of arachidonate release and other related events (46–52); it has been postulated that in neutrophils, although the effects on

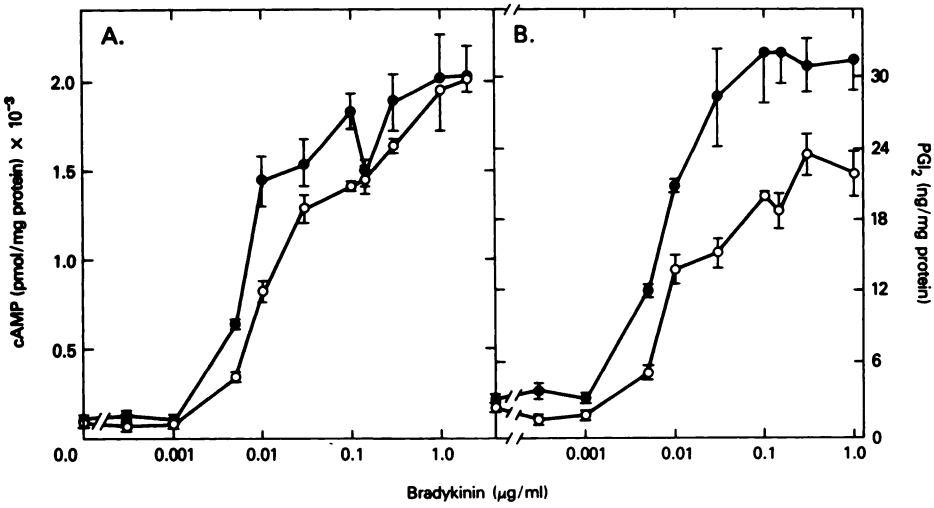

Fig. 5. Effect of pertussis toxin on bradykinin-induced cAMP accumulation and PGI_2 formation. Cells were incubated with (●) or without (○) pertussis toxin (1.56 μg) for 24 hr, then washed and incubated for 3 min with the indicated concentrations of bradykinin. A, cAMP accumulation; B, PGI_2 formation; each experiment was repeated three times with similar results. Data are expressed as mean ± standard error of triplicate incubations.

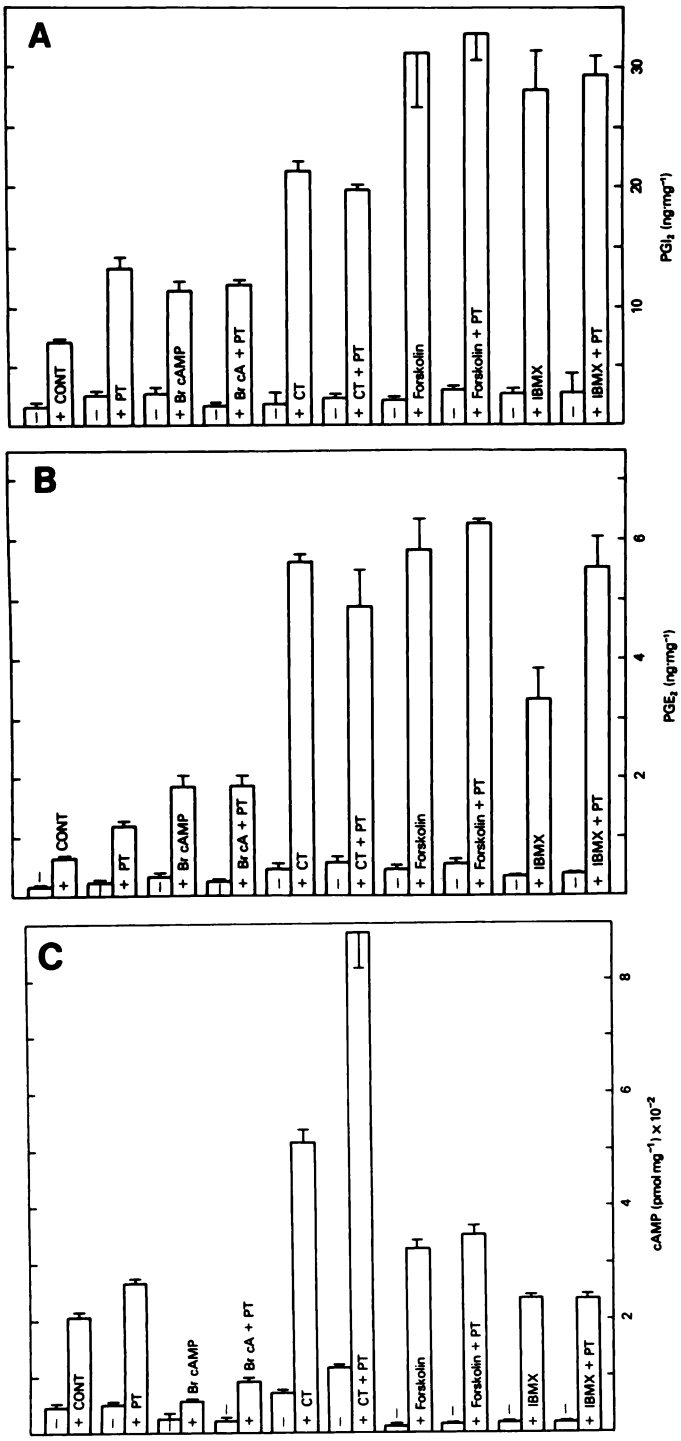

Fig. 6. Effect of agents that increase intracellular cAMP on bradykinin-induced and basal PGI_2 (A), PGE_2 (B), or cAMP (C) content. Cells were incubated for 24 hr with or without 1.72 μg of pertussis toxin (*PT*), 0.5 mM 8-bromo-cAMP, 1 μg/ml choleragen (*CT*), 10 μM forskolin, and/or 67 μM IBMX as indicated, then washed and incubated for 3 min with (+) or without (−) bradykinin (0.01 μg/ml). The medium was used for assay of PGI_2 (A) and PGE_2 (B), the cell layer for cAMP (C). This experiment was repeated three times with similar results. Data are expressed as mean ± standard error of triplicate incubations.

arachidonate metabolism result from modification of G_i, the events are not dependent on changes in cAMP content (46). From these studies as well as our data, it would thus appear that in different cell types ADP-ribosylation by pertussis toxin and/or changes in cAMP may have different effects on arachidonate metabolism. Further elucidation of the mechanisms of G protein action is necessary to understand the different effects of ADP-ribosylation in neutrophils and fibroblasts.

Discrimination between G_i and G_o on the basis of molecular weight is complicated by the fact that ADP-ribosylation alters the mobility of G proteins on SDS-polyacrylamide gels and the observations that proteolytic digestion products of G_i are similar in size to G_o (data not shown). In the present study, to determine whether G_o and/or G_i was present in the fibroblasts, membrane proteins were fractionated on the basis of size by SDS-polyacrylamide gel electrophoresis; the resolved proteins were transferred to nitrocellulose paper and the transferred material was incubated with antibodies exhibiting a high specificity for either G_i or G_o. Moreover, the presence of $G_{i\alpha 2}$ mRNA was confirmed by Northern analysis of fibroblast poly(A)$^+$ RNA using specific synthetic oligonucleotide probes for $G_{i\alpha 2}$. The results were compatible with the conclusion that the predominant toxin substrate is a G_i.

Pertussis toxin appears to enhance selectively the sensitivity to bradykinin; actions of other agents such as PGE_1 and isoproterenol were not affected. Thus, in human fibroblasts ADP-ribosylation of G_i appears to lead to an increase in basal cAMP, which in turn enhances the ability of bradykinin to increase prostaglandin formation. Based on this model, it can be postulated that enhanced effects of bradykinin after injection of pertussis toxin (27) may result from changes in effects of the agonist on arachidonate metabolism due to elevation in intracellular cAMP.

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