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Pertussis toxin abolishes angiotensin II-induced phosphoinositide hydrolysis and prostaglandin synthesis in rat renal mesangial cells

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Incubation of rat renal mesangial cells with angiotensin II (0.1 μM) resulted in transient breakdown of phosphatidylinositol 4,5-bisphosphate, rapid generation of diacylglycerol and phosphatidic acid, increased $^{45}Ca^{2+}$ influx, increased intracellular [Ca^{2+}] as measured by quin 2, and increased prostaglandin E_2 synthesis. All of these processes were markedly inhibited time- and dose-dependently by prior exposure of cells to pertussis toxin. In contrast, the effects of the ionophore A23187 on $^{45}Ca^{2+}$ influx and prostaglandin E_2 synthesis were not altered by the exposure of the cells to pertussis toxin. The action of the toxin was not associated with alterations in cellular concentrations of cyclic AMP. Incubation of membrane fraction of mesangial cells with pertussis toxin resulted in ADP-ribosylation of M_r-42000 protein. From all these results, it is likely that a G protein is involved in receptor-mediated signal transduction in renal mesangial cells.

INTRODUCTION

Receptor-stimulated hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) is thought to be an important mediator of cell activation through the generation of the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DG). Both of these products are likely to be involved in subsequent responses such as the mobilization of Ca^{2+} from endoplasmic reticulum and activation of protein kinase C [1]. However, the mechanism whereby hormone–receptor interaction leads to PIP_2 degradation remains unknown. Recent data in several systems suggest the involvement of GTP-binding proteins, the so-called G proteins [2–5]. Bacterial toxins, namely pertussis toxin and cholera toxin, have been suggested to interfere with receptor-mediated alterations of phospholipid metabolism by ADP-ribosylation of G proteins [2].

Previously we have shown that vasoconstrictors, such as angiotensin, [arginine]vasopressin and noradrenaline, caused a rapid breakdown of PIP_2 in rat renal mesangial cells, with concomitant production of DG and IP_3 [6, 7]. The activation of phospholipase C by these hormones further leads to an increased synthesis of prostaglandins (PG) in mesangial cells. The present study was carried out to gain information about the nature of the link between the occupation of the angiotensin II receptor and the activation of phospholipase C in rat mesangial cells. Our results show that pertussis toxin abolished the breakdown of PIP_2 and PGE_2 synthesis induced by angiotensin II in renal mesangial cells. It is suggested that a guanine-nucleotide-binding regulatory protein is somehow involved in receptor-mediated activation of phospholipase C in mesangial cells. Our results also provide further evidence for a causal link between stimulation of phospholipase C by vasoconstrictive hormones and increased prostaglandin synthesis.

EXPERIMENTAL

Cell culture

Rat mesangial cells were cultivated as described previously [8]. For all experiments the first passage of mesangial cells was used. The cells were grown in RPMI 1640 (Boehringer, Mannheim, Germany) supplemented with 10% (v/v) fetal bovine serum (Boehringer), penicillin (100 units/ml), streptomycin (100 μg/ml) (Boehringer) and bovine insulin (0.66 unit/ml) (Sigma). Tissue dishes (7 cm^2; Greiner, Nürtingen, Germany) were incubated in a humidified atmosphere in incubators in air/CO_2 (19:1).

Measurement of $^{45}Ca^{2+}$ uptake and $[Ca^{2+}]_i$

The uptake of $^{45}Ca^{2+}$ was measured 15, 30, 60 and 120 s after the addition of 4 μCi of $^{45}Ca^{2+}$/ml to the cells incubated in the presence of 2 mM-$CaCl_2$, and calculations were done as described previously [7].

$[Ca^{2+}]_i$ was measured by using the fluorescent calcium indicator quin 2. About 2×10^7 cells were incubated with 25 μM-quin 2/AM in RPMI 1640 for 20 min and then for 40 min with 4 vol. of the medium. After the incubation period, batches of cells (10^6) were washed twice and resuspended in Hepes-buffered saline (135 mM-NaCl, 5 mM-KCl, 1 mM-Na_2HPO_4, 1 mM-$MgCl_2$, 5 mM-glucose, 10 mM-Hepes, 10 μM-diethylenetriaminepentaacetic acid, pH 7.4) containing 1.3 mM Ca^{2+}. Fluorescence of quin-2-loaded cells was measured at 37 °C in a Shimadzu spectrofluorophotometer RF-510 with an excitation wavelength of 340 nm and emission wavelength of 492 nm. The fluorescence signal was calibrated at the end of each individual trace essentially as described by Tsien *et al.* [9].

Abbreviations used: PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol trisphosphate; DG, diacylglycerol; PG, prostaglandin; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; PA, phosphatidic acid.

Cell labelling

Medium was removed and the cells were incubated in minimal essential medium (Boehringer) supplemented with fatty-acid-free bovine serum albumin (0.3 mg/ml) (Sigma) and [^{14}C]arachidonic acid (0.25 μCi/ml) for 24 h. For the determination of phosphoinositides, cells were labelled with carrier-free [^{32}P]P_i (100 μCi/ml) in phosphate-poor minimal essential medium (50 μM-P_i) containing 10% dialysed fetal bovine serum for 24 h.

Lipid extraction and separation

After the prelabelling period, the medium was replaced by fresh medium either without or with 0.1 μM-angiotensin II. Incubations were terminated by rapid withdrawal of the incubation medium and the addition of 1 ml of ice-cold methanol to the cells. Lipids were extracted as described by Bligh & Dyer [10], and separated by t.l.c. as described previously [6]. In brief, neutral lipids were developed in one dimension with n-heptane/diethyl ether/acetic acid (75:25:4, by vol.). Polyphosphoinositides were separated, on thin-layer plates pretreated with 1% potassium oxalate containing 2 mM-EDTA, with chloroform/methanol/4 M-NH_4OH (9:7:2, by vol.). For separation of PA, chromatographs were run with the top phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (9:5:2:10, by vol.) [11]. Lipid standards were added as carriers and detected by iodine staining. The interesting lipids were scraped off and counted for radioactivity by liquid scintillation in water (Čerenkov counting) for ^{32}P-labelled lipids or with Econofluor (New England Nuclear) for ^{14}C-labelled lipids.

Prostaglandin, cyclic AMP and protein analysis

Culture dishes were washed twice with L-15 medium (Boehringer) and at the onset of the experiment 1 ml of fresh L-15 medium without or with 0.1 μM-angiotensin II was added, followed by incubation for 5 min. The medium was then withdrawn, frozen in liquid N_2 and stored at -80 °C until assay of PGE_2 by radioimmunoassay (New England Nuclear). Cells were dissolved in 1 M-NaOH and protein was determined by the method of Lowry *et al.* [12], with bovine serum albumin (Sigma) as standard. Cellular cyclic AMP was determined by radioimmunoassay (New England Nuclear).

Preparation of membranes, radiolabelling with [^{32}P]NAD^+ and electrophoresis

Mesangial cells were washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (Boehringer) and then incubated with a cold hypo-osmotic buffer consisting of 25 mM-Tris/HCl (pH 7.5), 5 mM-$MgCl_2$, 1 mM-EGTA, 1 mM-dithiothreitol and 50 units of the proteinase inhibitor aprotinin/ml. After 15 min on ice, the cells were scraped from the dish with a rubber policeman, and the suspension was centrifuged at 10000 *g* for 3 min. The supernatant was aspirated, and the pellet sonicated in 100 mM-potassium phosphate buffer (pH 7.5) containing 5 mM-$MgCl_2$, 20 mM-thymidine and 1 mM-ATP on ice for 15 s with a Branson B 15 sonifier (setting 5.0). The sonicated homogeneous membrane suspensions (about 100 μg of protein) were then incubated with 10 μM-[*adenylate*-^{32}P]NAD^+ and 10 μg of pertussis toxin/ml in a final volume of 100 μl. After 20 min of incubation at 30 °C, the membranes were centrifuged at 10000 *g* for 3 min, dissolved in Laemmli sample buffer [13] and incubated for 5 min at 96 °C. Thereafter a portion of each sample was submitted to horizontal polyacrylamide-gel electrophoresis on ultrathin (0.5 mm) polyacrylamide gels, containing a linear pore gradient (4–22.5% acrylamide). The gel was then autoradiographed at −80 °C for 24 h with Kodak X-Omat AR film and an enhancer foil (Dupont Cronex Quanta IIF).

Angiotensin II-binding assay

Binding experiments were done with dissociated cells scraped off their flasks exactly as described by Foidart *et al.* [14], as follows: ^{125}I-angiotensin II was added at 0.5 nM and incubated with 30–40 μg of cellular proteins per tube at room temperature for 40 min. At the end of the incubation, bound radioactivity was separated by filtration through a Millipore filter (0.22 μm; GS). A portion of the cell suspension was used for protein determination. Specific binding was calculated by subtracting the binding in the presence of 5 mM unlabelled angiotensin II from total binding and expressed as fmol of bound angiotensin II/mg of cell protein.

Chemicals

The materials and their sources were as follows: lipid standards, angiotensin II (Sigma Chemical Co., St. Louis, MO, U.S.A.); pertussis toxin (islet-activating protein) (List Biological Laboratories, Campbell, CA, U.S.A.); carrier-free [^{32}P]P_i, $^{45}CaCl_2$ and [^{14}C]arachidonic acid (Amersham International, Braunschweig, Germany); [*adenylate*-^{32}P]NAD^+ (28 Ci/mmol) and [*tyrosine*-^{125}I]-angiotensin II (New England Nuclear, Boston, MA, U.S.A.); all other chemicals were from Merck, Darmstadt, Germany.

RESULTS

Inhibition of angiotensin II-induced PGE_2 synthesis by pertussis toxin

The basal rate of PGE_2 synthesis in cultured mesangial cells was 1.8 ng/5 min per mg of protein. Addition of angiotensin II (0.1 μM) increased PGE_2 release by 348%, to 6.3 ng/5 min per mg of protein. Preincubation of mesangial cells with pertussis toxin for 15 h dose-dependently attenuated the angiotensin II-evoked PGE_2 synthesis, as shown in Fig. 1(*a*).The half-maximally inhibitory concentration of pertussis toxin was about 1 ng/ml. The ability of pertussia toxin to inhibit angiotensin II-induced PGE_2 synthesis also depended on the time of preincubation of the cells with the toxin. As shown in Fig. 1(*b*), the inhibitory effect of pertussis toxin reached a maximal value after about 15 h of preincubation time. The onset of action of pertussis toxin was rapid, and the half-maximally inhibitory effect was reached already after 1–2 h. For all further experiments we therefore chose a preincubation time of 15 h at a concentration of 100 ng of pertussis toxin/ml to assure a complete inhibition of angiotensin II-induced effects. In contrast with the results obtained with angiotensin II, pertussis toxin did not inhibit the PGE_2 synthesis evoked by the addition of exogenous arachidonic acid (10 μM) (results not shown). This clearly indicates that pertussis toxin did not interfere with cyclo-oxygenase activity. Pertussis toxin also did not inhibit the PGE_2 synthesis stimulated by the calcium ionophore A23187 (1 mM):

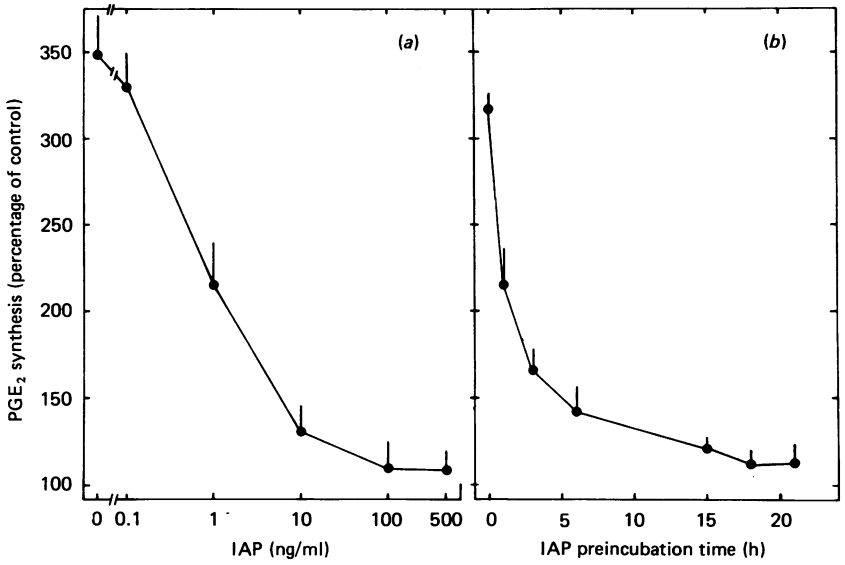

Fig. 1. Time- and dose-dependency of inhibition of angiotensin II-induced PG synthesis by pertussis toxin

Mesangial cells were incubated for 5 min with angiotensin II (0.1 μM), and PG synthesis is expressed as a percentage of control (cells stimulated with vehicle alone). Values are the means ± S.E.M. for five experiments. (*a*) Cells preincubated for 15 h with the indicated concentration of pertussis toxin (islet-activating protein, IAP). (*b*) Cells preincubated with 100 ng of pertussis toxin/ml for the indicated time period.

1.5 ± 0.2 ng/5 min per mg of protein (control), 9.6 ± 1.2 ng/5 min per mg of protein (A23187), 8.7 ± 1.1 ng/5 min per mg of protein (pertussis toxin) (means ± S.E.M., $n = 3$).

Inhibition of angiotensin II-induced increase in $^{45}Ca^{2+}$ influx and $[Ca^{2+}]_i$ by pertussis toxin

The basal $^{45}Ca^{2+}$ influx as calculated from the slope of the regression lines was 2834 ± 131 c.p.m./min per mg of cell protein (mean ± S.E.M., $n = 6$). Addition of angiotensin II (0.1 μM) at the same time as $^{45}Ca^{2+}$ increased the influx to 146% of control value (Table 1). Preincubation with pertussis toxin totally abolished the angiotensin II-induced increase in $^{45}Ca^{2+}$ influx, whereas pertussis toxin alone did not alter the basal $^{45}Ca^{2+}$ influx. In contrast with the results with angiotensin II, the effects of A23187 (1 μM) on $^{45}Ca^{2+}$ influx was not influenced by prior exposure of mesangial cells to pertussis toxin (Table 1).

The basal $[Ca^{2+}]_i$ as measured with the fluorescent calcium indicator quin-2 was 199 ± 4.0 nM (mean ± S.E.M., $n = 17$). Angiotensin II (0.1 μM) increased this transiently to 382 ± 41 nM (mean ± S.E.M., $n = 9$). Pretreatment with pertussis toxin did not alter the basal $[Ca^{2+}]_i$ (188 ± 12 nM; mean ± S.E.M., $n = 3$), but completely

Table 1. Effect of pertussis toxin on angiotensin II- and ionophore-induced $^{45}Ca^{2+}$ influx

Mesangial cells were preincubated with pertussis toxin (100 ng/ml) or vehicle for 15 h. After this preincubation period, $^{45}Ca^{2+}$ influx was measured as outlined in the Experimental section. Results are means ± S.E.M. (n = 6).

Addition	$^{45}Ca^{2+}$ influx (c.p.m./min per mg of protein)
Control	2834 ± 131
Pertussis toxin	2730 ± 265
Angiotensin II (100 nM)	4129 ± 419
Pertussis toxin + angiotensin II (100 nM)	2581 ± 87
A23187 (1 μM)	7493 ± 954
Pertussis toxin + A23187 (1 μM)	7052 ± 822

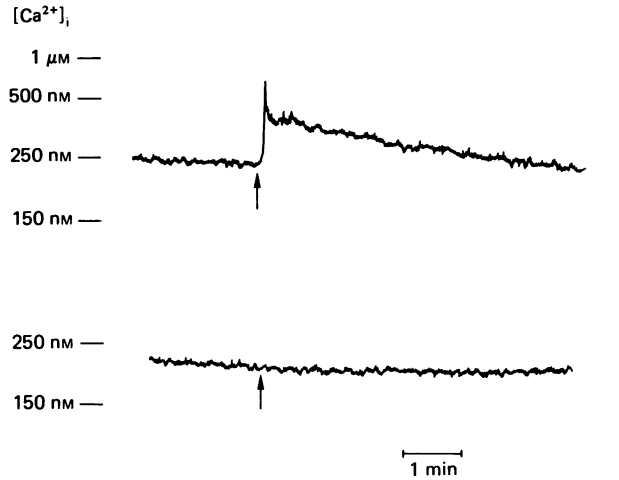

Fig. 2. $[Ca^{2+}]_i$ in mesangial cells as indicated by quin 2 fluorescence

$[Ca^{2+}]_i$ was calculated from changes in quin 2 fluorescence. The upper part of the Figure shows the effect of angiotensin II (0.1 μM) on $[Ca^{2+}]_i$. The lower part of the Figure shows that pretreatment of mesangial cells with pertussis toxin (100 ng/ml; 15 h) totally abolished the antiotensin II-induced increase in $[Ca^{2+}]_i$. Arrow indicates addition of angiotensin II.

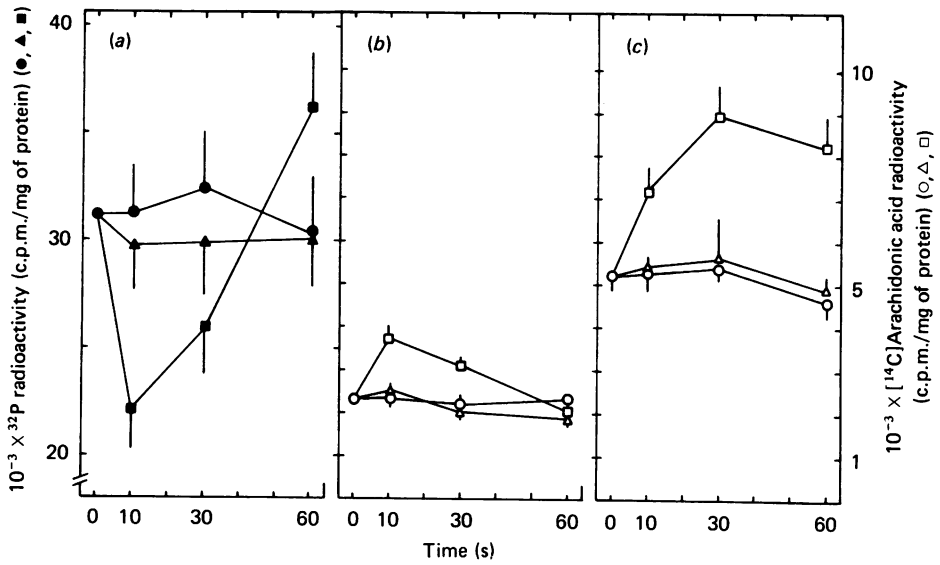

Fig. 3. Time course of ^{32}P radioactivity in PIP_2 (*a*) and of [^{14}C]arachidonic acid radioactivity in DG (*b*) and PA (*c*)

●, ○, Control values; ■, □, cells stimulated with angiotensin II (0.1 μM); ▲, △, cells pretreated with pertussis toxin (100 ng/ml; 15 h) and stimulated with angiotensin II (0.1 μM). Values are means ± S.E.M. (n = 5).

abolished the angiotensin II-induced increase in $[Ca^{2+}]_i$ (Fig. 2).

Inhibition of angiotensin II-induced activation of phospholipase C by pertussis toxin

As we have shown previously, angiotensin II activates a phospholipase C in mesangial cells [7]. In cells prelabelled with [^{32}P]P_i, angiotensin II caused a breakdown of PIP_2, and this degradation of PIP_2 could be inhibited by prior treatment of the cells with pertussis toxin (Fig. 3*a*). In accordance with these results, we also found that, in cells prelabelled with [^{14}C]arachidonic acid, pertussis toxin completely blocks the angiotensin II-induced increase in radioactivity in DG and also (Figs. 3*b* and 3*c*). These data indicate that pertussis toxin is able to prevent an activation of phospholipase C in mesangial cells stimulated with angiotensin II.

Specific binding of [tyrosine-^{125}I]angiotensin II to pertussis-toxin-treated and non-treated cells

To exclude the possibility that the pertussis-toxin-induced attenuation of the angiotensin II effects is due to a decreased density or affinity of receptors on mesangial cells, we determined the specific binding of ^{125}I-angiotensin II. This binding to intact mesangial cells was plotted as a function of the concentration of the labelled peptide hormone. A linear curve was obtained, corresponding to one group of receptor sites. The maximal number of binding sites (39 fmol/mg of protein) and the apparent dissociation constant, K_d (2.1 nM) were calculated. There was no significant difference either in the number of binding sites or in their affinities for angiotensin II between control and pertussis-toxin-treated cells.

Effect of angiotensin II on cellular cyclic AMP contents in pertussis-toxin-treated and non-treated cells

One of the potential effects of treatment with pertussis toxin is to enhance cyclic AMP accumulation in response to stimulatory agonists [15]. We therefore tested for a possible involvement of altered cyclic AMP metabolism

Table 2. Effect of angiotensin II on cyclic AMP concentrations in pertussis-toxin-treated and non-treated cells

Mesangial cells were preincubated with pertussis toxin (100 ng/ml) or vehicle for 15 h. After this preincubation period, cellular cyclic AMP concentrations were measured as outlined in the Experimental section. Results are mean ± S.E.M. (n = 6).

Addition	Cyclic AMP (pmol/mg of protein)
Control	20.8 ± 3.1
Pertussis toxin	17.4 ± 3.3
Angiotensin II (100 nM)	23.3 ± 4.2
Pertussis toxin + angiotensin II (100 nM)	17.2 ± 3.8

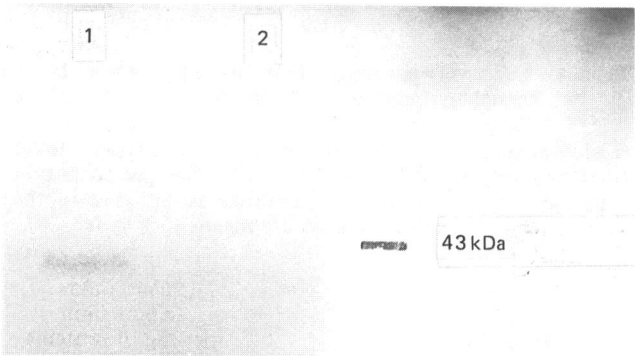

Fig. 4. Pertussis-toxin-catalysed incorporation of [^{32}P]NAD^+ into mesangial cell membranes

ADP-ribosylation of mesangial cell membranes and SDS/polyacrylamide-gel electrophoresis and autoradiography were carried out as described in the Experimental section. Lane 1, membrane preparation + pertussis toxin (10 μg/ml); lane 2, no pertussis toxin added.

in the inhibition of angiotensin II-evoked PGE_2 synthesis and PIP_2 degradation induced by pertussis toxin. The cellular cyclic AMP content in control cells was 20.8 ± 3.1 pmol/mg of protein (mean $\pm$ S.E.M.; $n = 6$), and preincubation with 100 ng of pertussis toxin/ml for 15 h did not change this significantly (Table 2). When mesangial cells were stimulated for 5 min with angiotensin II, cellular cyclic AMP content was not significantly altered whether the cells were pretreated with pertussis toxin or not.

Pertussis toxin ADP-ribosylated a 42000-Da membrane protein

Exposure of a variety of cells to pertussis toxin resulted in ADP-ribosylation of the α-subunit of G_i, a protein of 41000 Da [15]. In mesangial cells a single protein of M_r 42000 was ADP-ribosylated by pertussis toxin, as shown in Fig. 4.

DISCUSSION

The present study demonstrates that treatment of mesangial cells with pertussis toxin completely abolished angiotensin II-induced activation of phospholipase C, as measured by the lack of PIP_2 degradation and subsequent increase in DG and PA. This inhibition of phospholipase C activity was accompanied by a total inhibition of Ca^{2+} mobilization. $^{45}Ca^{2+}$ influx, as well as increases in $[Ca^{2+}]_i$ measured with quin 2, were totally abolished, indicating that pertussis toxin interfered with proccesses proximal to calcium gating. Comparable results were reported by Nakamura & Ui [2] for mast cells, by Volpi *et al.* for neutrophilis [16] and by Brandt *et al.* [4] for human leukaemia (HL-60) cells. On the other hand, Murayama & Ui [17] reported that pertussis toxin did not inhibit the thrombin-induced breakdown of inositol phospholipids in 3T3 fibroblasts.

Pertussis toxin is an ADP-ribosyltransferase with a 41000-Da protein as one of the specific substrates [15]. Fig. 4 shows that also in mesangial cells pertussis toxin ADP-ribosylated a membrane protein of about 42000 Da. If this protein were the α-subunit of G_i, the inhibitory regulatory protein of adenylate cyclase, more cyclic AMP should accumulate in pertussis-toxin-treated cells than in control cells. Since Hassid [18] has shown that increased concentrations of cyclic AMP are able to inhibit bradykinin-stimulated PG synthesis in MDCK cells by interering with acylhydrolase and cyclo-oxygenase activity, this would also explain the inhibitory effect of pertussis toxin on angiotensin II-induced PG synthesis. The cyclic AMP content of pertussis-toxin-treated mesangial cells, however, did not differ from that in non-treated cells whether they were stimulated with angiotensin II or not. This indicates that the inhibition of angiotensin II-induced PG production by pertussis toxin was not mediated by increases in cyclic AMP.

A second possibility for the mode of action of pertussis toxin might be an alteration of the binding activity of membrane receptors for angiotensin II. This possibility, however, is unlikely, because there was no significant difference between pertussis-toxin-treated and non-treated cells either in the number of binding sites or in their affinities for angiotensin II.

A third, and most likely, possibility is that pertussis toxin modifies directly the signal transduction from angiotensin II receptors to PG synthesis. Although the detailed mechanism by which angiotensin II stimulates PG synthesis is not clear, there is evidence that this process is Ca^{2+}-dependent and is mediated by a subsequent activation of phospholipase C and protein kinase C [7]. The treatment of mesangial cells with pertussis toxin blocked the action of angiotensin II to increase Ca^{2+} influx as well as to increase $[Ca^{2+}]_i$ (Fig. 2) and also abolished angiotensin II-induced PG synthesis. In contrast, pertussis toxin did not alter the action of the ionophore A23187 on Ca^{2+} influx and PG synthesis in mesangial cells. Thus the processes subsequent to Ca^{2+} entry appear to be insensitive to pertussis toxin, at least in mesangial cells.

Cockcroft & Gomperts [3] have suggested an involvement of a GTP-binding protein in coupling of receptor to phospholipase C, which mediates the Ca^{2+}-dependent histamine release from mast cells. Our results showed that phospholipase C stimulation by angiotensin II resulting in degradation of PIP_2 is sensitive to pertussis toxin. In analogy to mast cells, neutrophils and HL-60 cells [2, 4, 16], this might indicate that a GTP-binding protein is involved in coupling of the angiotensin II receptor to phospholipase C activation in renal mesangial cells. Our results also provide evidence that activation of phospholipase C is an essential step in the angiotensin II-induced liberation of arachidonic acid and subsequent synthesis of PG. These findings strengthen the hypothesis that there is a causal relationship between agonist-induced activation of phospholipase C and subsequent increased PG synthesis in mesangial cells.

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