Vol. 152, No. 1, 1988
April 15, 1988

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 285-293

DIFFERENT SENSITIVITY TO PHORBOL ESTERS AND PERTUSSIS TOXIN OF BOMBESIN- AND PLATELET-DERIVED GROWTH FACTOR-INDUCED, PHOSPHOLIPASE C-MEDIATED HYDROLYSIS OF PHOSPHOINOSITIDES IN NIH/3T3 CELLS[1]

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Received March 1, 1988

SUMMARY: Incubation of the serum-deprived cultures of NIH/3T3 cells with bombesin or platelet-derived growth factor (PDGF) induced the phospholipase C-mediated hydrolysis of phosphoinositides. Protein kinase C-activating 12-O-tetradecanoylphorbol 13-acetate (TPA) and pertussis toxin inhibited the bombesin-induced phospholipase C reactions. AlF_4^-, a direct activator of GTP-binding proteins (G proteins), also induced the phospholipase C reactions and TPA inhibited the AlF_4^--induced reactions. These results suggest that a pertussis toxin-sensitive G protein is involved in the coupling of the bombesin receptor to the phospholipase C and that the coupling of the G protein to the phospholipase C is inhibited by protein kinase C. In contrast, neither TPA nor pertussis toxin inhibited the PDGF-induced phospholipase C reactions, indicating that a pertussis toxin-sensitive G protein is not involved in the coupling of the PDGF receptor to the phospholipase C and that this coupling is insensitive to protein kinase C. These results suggest that the regulatory mechanism of the PDGF receptor for the phospholipase C activation is different from that of the bombesin receptor.

The phospholipase C-mediated hydrolysis of phosphoinositides in a receptor-linked manner produces diacylglycerol and inositol

1. This investigation was supported in part by research grants from the Scientific Research Fund of the Ministry of Education, Science and Culture, Japan (1987), Investigation Committee on Abnormalities in Hormone Receptor Mechanisms and Caldiovascular Diseases (62A-1), the Ministry of Health and Welfare, Japan (1987), the Yamanouchi Foundation for Research on Metabolic Disorders (1987) and the Research Program on Cell Calcium Signals in the Cardiovascular System (1987).

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The abbreviations used are: G protein, GTP-binding protein; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; TPA, 12-O-tetradecanoylphorbol 13-acetate; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; IP_3, inositol trisphosphate.

0006-291X/88 $1.50

trisphosphates, which serve as the messengers for the protein kinase C activation and for the intracellular Ca^{2+} mobilization, respectively (for reviews, see Refs. 1 and 2). Evidence is accumulating that a G protein is involved in the coupling of the receptors to the phospholipase C (for a review, see Ref. 3). Although the G protein involved in this coupling has not been identified, a pertussis toxin-sensitive G protein(s) has been shown to be one of coupling factors in some types of cells (3). Another line of evidence indicates that the receptor-linked phospholipase C reactions are inhibited by protein kinase C in several receptor systems (4, for a review, see Ref. 5). It has recently been shown that protein kinase C inhibits the coupling of the G protein to the phospholipase C (6-8). This protein kinase C-induced inhibition of the receptor-linked phospholipase C reactions is considered to be related to a desensitization mechanism of receptors by analogy with the adenylate cyclase system (for a review, see Ref. 9).

Bombesin and PDGF are potent growth factors for several cell types (10-15). These growth factors induce the phospholipase C reactions in mouse 3T3 cells (16-21). However, the regulatory mechanisms of the bombesin and PDGF receptors for the phospholipase C activation have not been clarified. The present studies were undertaken to investigate whether a pertussis toxin-sensitive G protein is involved in the coupling of the bombesin and PDGF receptors to the phospholipase C and whether these couplings are sensitive to protein kinase C. This paper describes that the regulatory mechanism of the PDGF receptor for the activation of phospholipase C is different from that of the bombesin receptor.

EXPERIMENTAL PROCEDURES

Materials and Chemicals——NIH/3T3 cells were kindly supplied by Japan Cancer Research Resources Bank which originally obtained them from Dr. S.A. Aaronson (National Cancer Institute, Maryland,

U.S.A.). Stock cultures of NIH/3T3 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air in DMEM containing 10% calf serum. Bombesin and highly purified PDGF were purchased from Peptide Institute Inc. (Osaka, Japan) and Collaborative Research Inc. (Massachusetts, U.S.A.), respectively. Pertussis toxin was a generous gift from Dr. T. Katada (Tokyo Institute of Technology, Yokohama, Japan) and Dr. M. Ui (Tokyo University, Tokyo, Japan). TPA was from CCR Inc.. myo-[2-^{3}H]Inositol (19-20 Ci/mmol) and [^{32}P]NAD (800 Ci/mmol) were from Amersham and New England Nuclear, respectively. Plasma-derived serum was prepared from human blood as described (22). Other materials and chemicals were obtained from commercial sources.

Assay for the Formation of Inositol Phosphates——NIH/3T3 cells (1 x 10^5) were seeded into 35-mm diameter dishes in 2 ml of DMEM containing 10% calf serum. After the cells were incubated for 18 h, the cells were labelled with myo-[2-^{3}H]inositol (3 μCi/dish) in the same medium for 40 h. The cells were then incubated in inositol-free DMEM containing 2% plasma-derived serum and myo-[2-^{3}H]inositol (3 μCi/dish) for 20 h to render the cells quiescent. Where indicated, the cells were incubated with various doses of pertussis toxin for the last 6 h during this incubation period. The labelled cells were washed once with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid at pH 7.4 containing 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM glucose, 0.1% bovine serum albumin and 20 mM LiCl. After the cells were preincubated with or without TPA in the same solution for 20 min, the cells were stimulated by bombesin or PDGF. The reaction was terminated by 15% trichloroacetic acid. Inositol phosphates were extracted and separated by batch chromatography on Dowex AG1-X8 formate form resins as described (19,23).

ADP-ribosylation of Membrane Proteins by Pertussis Toxin——NIH/3T3 cells (6 x 10^5) were seeded into 100-mm diameter dishes in 12 ml of DMEM containing 10% calf serum, refed with the same medium for 18 h, and incubated for further 40 h. The cells were then incubated in DMEM containing 2% plasma-derived serum for 20 h. The cells were pretreated with 1 μg/ml of pertussis toxin or vehicle for the last 6 h during this incubation period. The cells were washed twice with ice-cold phosphate-buffered saline and scraped off with a rubber policeman in 2 ml of 25 mM Tris/HCl at pH 7.5 containing 5 mM $MgCl_2$ and 1 mM phenylmethylsulfonyl fluoride. The cells were disrupted with N_2 cavitation for 20 min at 350 psi. The disrupted cells were centrifuged at 100 x g for 5 min to remove undisrupted cells. The supernatant was further centrifuged at 100,000 x g for 1 h. The pellet was resuspended in 20 mM Tris/HCl at pH 8.0 containing 1 mM EDTA, 1 mM dithiothreitol, 25 mM NaCl and 1% sodium cholate at a final concentration of 10 mg of protein/ml, and incubated with constant shaking for 60 min at 4°C. The mixture was centrifuged at 100,000 x g for 1 h. The extract (50 μg of protein) was ADP-ribosylated by the preactivated A-protomer of pertussis toxin with [^{32}P]NAD as a ADP-ribose donor under the conditions described (24). The proteins were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide) as specified by Laemmli (25). After the gel was dried, an autoradiograph was made using a Kodak X-Omat film.

Determinations Protein was determined by the method of Lowry et al. (26) with bovine serum albumin as a standard protein. Radioactivity of ^{3}H-labelled samples was determined using a Beckman liquid scintillation system, Model LS3801.

RESULTS

Incubation of the serum-deprived NIH/3T3 cells with bombesin or PDGF induced the phospholipase C reactions in a time-dependent manner as estimated by the formation of IP_1, IP_2 and IP_3 as shown in Fig. 1. The time courses of the formation of these inositol phosphates induced by bombesin and PDGF were almost the same.

TPA, known to be a potent protein kinase C activator (1), inhibited the bombesin-induced phospholipase C reactions as shown in Fig. 1. The doses of TPA necessary for the maximal and half maximal inhibitions were 20 nM and 1 nM, respectively (data not shown). These values are the same as the doses necessary for the activation of protein kinase C (1). Phorbol 12,13-dibutyrate, another protein kinase C-activating phorbol ester (1), inhibited the bombesin-induced phospholipase C reactions, but 4α-phorbol

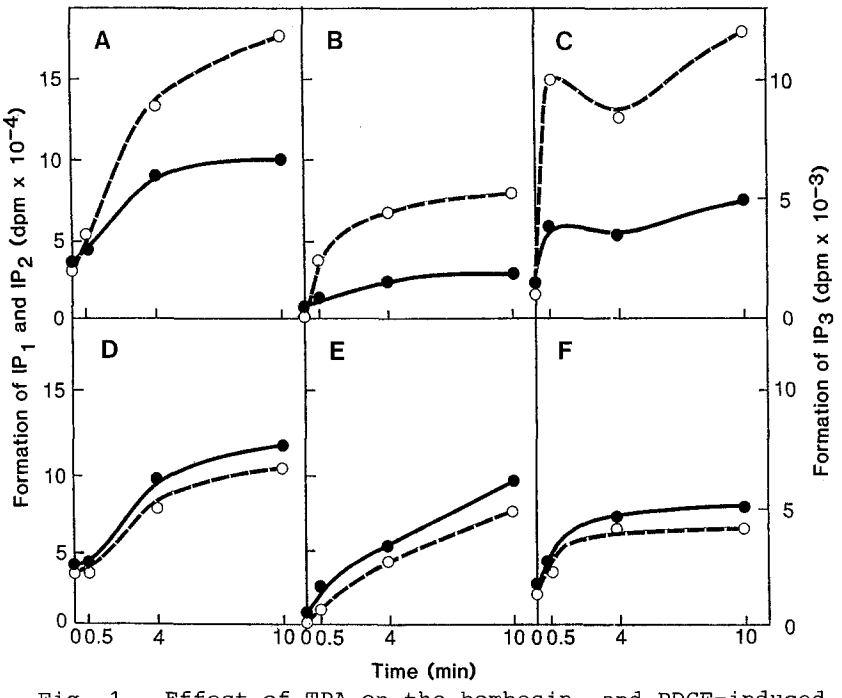

Fig. 1. Effect of TPA on the bombesin- and PDGF-induced formation of inositol phosphates. NIH/3T3 cells prelabelled with [^{3}H]inositol were preincubated in the presence of TPA (100 nM) or vehicle for 20 min and then stimulated by bombesin (1 μM) or PDGF (4 ng/ml) for the indicated periods of time. (A-C), stimulated by bombesin; (D-F), stimulated by PDGF. (A,D), IP_1; (B,E), IP_2; (C,F), IP_3. (○—○), in the absence of TPA; (●—●), in the presence of TPA. Each value is the mean of triplicate determinations.

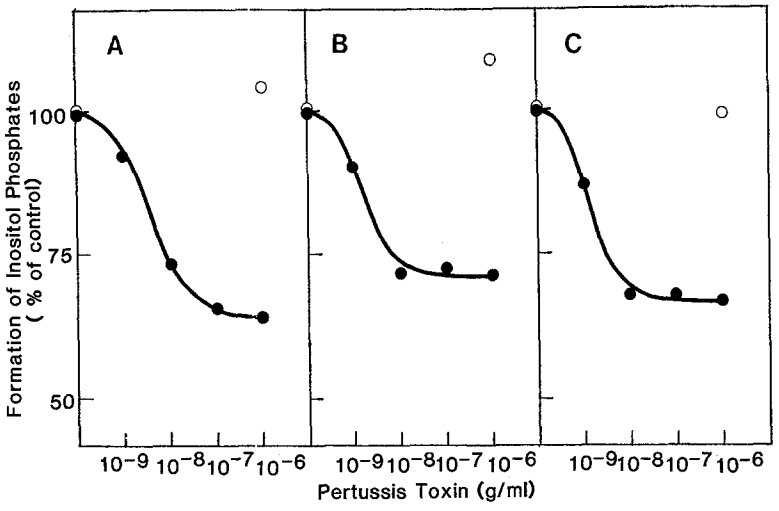

Fig. 2. Effect of pertussis toxin on the bombesin- and PDGF-induced formation of inositol phosphates. NIH/3T3 cells prelabelled with [^{3}H]inositol were preincubated with various doses of pertussis toxin for 6 h and then stimulated by bombesin (1 μM) or PDGF (4 ng/ml) for 4 min. (A), IP_1; (B), IP_2; (C), IP_3. (●—●), stimulated by bombesin; (○), stimulated by PDGF. Results are expressed as % of control responses to bombesin (IP_1, 1.31 x 10^5 dpm; IP_2, 7.14 x 10^4 dpm; IP_3, 7.74 x 10^3 dpm) and PDGF (IP_1, 7.27 x 10^4 dpm; IP_2, 4.21 x 10^4 dpm; IP_3, 4.22 x 10^3 dpm) in the absence of pertussis toxin, respectively. Each value is the mean of triplicate determinations.

12,13-didecanoate, which is inactive for protein kinase C (1), was ineffective in this capacity (data not shown). In contrast, TPA did not inhibit the PDGF-induced phospholipase C reactions under the same conditions.

Pertussis toxin partially inhibited the bombesin-induced phospholipase C reactions in a dose-dependent manner as shown in Fig. 2. This toxin ADP-ribosylated a protein with a Mr of about 40,000 as shown in Fig. 3. In contrast, pertussis toxin did not

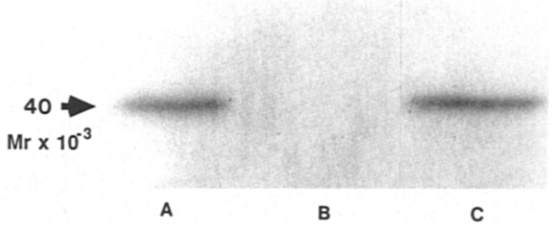

Fig. 3. The ADP-ribosylation of membrane proteins. NIH/3T3 cells were pretreated with pertussis toxin or vehicle for 6 h. The membrane proteins were prepared and ADP-ribosylated by the preactivated pertussis toxin with [^{32}P]NAD. (lane A), pretreated without the toxin; (lane B), pretreated with the toxin (1 μg/ml); (lane C), rat brain G_i as a marker protein. The data are typical of three independent experiments.

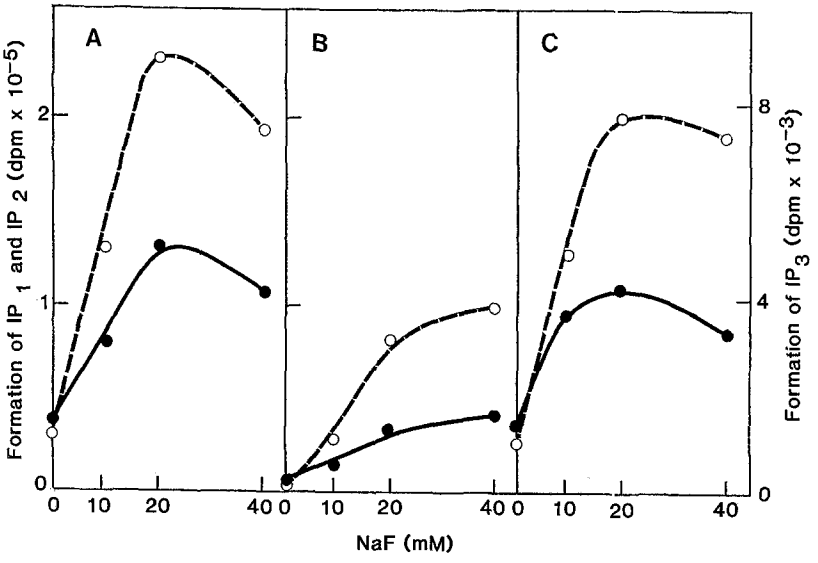

Fig. 4. Inhibition by TPA of the AlF_4^--induced formation of inositol phosphates. NIH/3T3 cells prelabelled with [^{3}H]inositol were preincubated in the presence of TPA (100 nM) or vehicle for 20 min and then stimulated by various doses of NaF in the presence of 10 μM $AlCl_3$ for 30 min. (A), IP_1; (B), IP_2; (C), IP_3; (○—○), in the absence of TPA; (●—●), in the presence of TPA. Each value is the mean of triplicate determinations.

inhibit the PDGF-induced phospholipase C reactions under the same conditions.

NaF plus $AlCl_3$ is known to activate G proteins directly presumably through an AlF_4^- form (3). Incubation of the cells with NaF plus $AlCl_3$ instead of the growth factors induced the formation of IP_1, IP_2 and IP_3 in a dose-dependent manner as shown in Fig. 4. TPA again inhibited the phospholipase C reactions induced by NaF plus $AlCl_3$.

DISCUSSION

We have shown in this paper that the bombesin-induced phospholipase C-mediated hydrolysis of phosphoinositides is inhibited by pertussis toxin in NIH/3T3 cells. This inhibition by the toxin is incomplete. The exact reason for this partial inhibition is not known, but this result suggests that a pertussis toxin-sensitive G protein is at least partly involved in the coupling of the

bombesin receptor to the phospholipase C in this cell type. This suggestion is supported by another line of evidence that AlF_4^-, known to activate G proteins directly (3), induces the phospholipase C reactions. Zachary et al. (27) have shown that the bombesin-induced phospholipase C reactions are not inhibited by pertussis toxin in Swiss 3T3 cells. The exact reason for the inconsistency between their results and ours is not known, but it may be due to differences in the cell types or assay conditions.

We have also shown here that the bombesin- and AlF_4^--induced phospholipase C reactions are inhibited by TPA. The inhibition by TPA of the bombesin-induced phospholipase C reactions have been reported in Swiss 3T3 cells (28) and the inhibition by TPA of the AlF_4^--induced reactions in CCL39 Chinese hamster lung fibroblasts (29). Our present results together with these earlier observations suggest that protein kinase C inhibits the coupling of the G protein to the phospholipase C.

In contrast to the action of bombesin, the PDGF-induced phospholipase C reactions are inhibited neither by pertussis toxin nor by TPA under the conditions where the bombesin-induced phospholipase C reactions are inhibited by these agents. This result suggests that a pertussis toxin-sensitive G protein is unlikely to be involved in the coupling of the PDGF receptor to the phospholipase C and that protein kinase C does not inhibit the coupling of the PDGF-receptor to the phospholipase C. However, it remains to be clarified whether or not a pertussis toxin-insensitive G protein is involved in the coupling of the PDGF receptor to the phospholipase C. It has been shown that the receptors which interact with G proteins, such as rhodopsin (30), the β-adrenergic (31) and the muscarinic acetylcholine (32) receptors, contain seven transmembrane segments while the PDGF receptor has only one transmembrane segment (33). The present results together with the

information on the receptor structure suggest that the mechanism of the PDGF receptor for the phospholipase C activation is different from that of the bombesin receptor. Further investigation is essential for understanding how the PDGF receptor regulates the phospholipase C.

ACKNOWLEDGEMENT

We are grateful to Junko Yamaguchi for her skillful secretarial assistance.

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