Inhibition of prostaglandin E₂-induced phosphoinositide metabolism by phorbol ester in bovine adrenal chromaffin cells

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In bovine adrenal chromaffin cells, prostaglandin E₂ (PGE₂) stimulates the formation of inositol phosphates and Ca²⁺ mobilization through its specific receptor [Yokohama, Tanaka, Ito, Negishi, Hayashi & Hayaishi (1988) *J. Biol. Chem.* **263**, 1119–1122]. Here we show that PGE₂-induced phosphoinositide metabolism was blocked by pretreatment with 12-*O*-tetradecanoylphorbol 13-acetate (TPA). Using intact cells, we also examined the inhibitory effect of TPA on the individual steps of the activation process of phosphoinositide metabolism. The inhibition was observed within 1 min and complete by 10 min after addition of 1 μM-TPA, and half-maximal inhibition by TPA occurred at 20 nM. TPA prevented Ca²⁺ mobilization induced by PGE₂, but not by the Ca²⁺ ionophore ionomycin. The inactive phorbol ester 4α-phorbol 12,13-didecanoate did not inhibit the formation of inositol phosphates and Ca²⁺ mobilization by PGE₂. TPA treatment affected neither the high-affinity binding of [³H]PGE₂ to intact cells and membrane fractions nor the ability of guanosine 5'-[γ-thio]triphosphate to decrease the binding in membrane fractions. TPA also abolished phosphoinositide metabolism induced by muscarinic-receptor activation. NaF plus AlCl₃ and ionomycin caused the accumulation of inositol phosphates, probably by directly activating a GTP-binding protein(s) and phospholipase C respectively; neither accumulation was inhibited by TPA treatment. These results suggest that protein kinase C serves as a feedback regulator for PGE₂-induced phosphoinositide metabolism. The site of action of TPA appears to be distal to the coupling of the receptor to GTP-binding protein, but on a component(s) specific to the agonist-induced phosphoinositide metabolism.

INTRODUCTION

It is well established that prostaglandin E₂ (PGE₂) exerts its physiological and pharmacological effects through binding to specific cell-surface receptors and stimulation of adenylate cyclase [1,2]. However, we have recently demonstrated that the PGE receptor in bovine adrenal medulla may be functionally associated with a pertussis-toxin-insensitive GTP-binding protein (G-protein) and that this receptor is not coupled to the adenylate cyclase system [3]. More recently, we also showed that exposure of cultured bovine chromaffin cells to PGE₂ results in a drastic increase in catecholamine release in the presence of ouabain, whereby PGE₂ stimulates phosphoinositide metabolism and Ca²⁺ mobilization [4]. These results taken together suggest that the PGE receptor may be coupled to phospholipase C via a pertussis-toxin-insensitive G-protein in bovine adrenal medulla. This notion seems to be reasonable, in the light of recent evidence that a G-protein may be involved in the coupling of receptors to the phospholipase C in isolated membranes [5–8], by analogy with the adenylate cyclase system. On the other hand, two groups reported that a rise in cytosolic free Ca²⁺ concentration ([Ca²⁺]_i) activates phospholipase C directly in chromaffin cells,

with the resultant formation of inositol phosphates [9,10].

The products of phosphoinositide metabolism, inositol trisphosphate (InsP₃) and 1,2-diacylglycerol, have been demonstrated to release Ca²⁺ from the endoplasmic reticulum and to activate protein kinase C respectively; this kinase, in turn, phosphorylates various cellular proteins [11,12]. Phorbol esters, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA), are known to activate protein kinase C directly by replacing the requirement for 1,2-diacylglycerol [13]. Recent studies, however, have demonstrated that agonist-induced phosphoinositide metabolism and Ca²⁺ mobilization are inhibited by pretreatment with phorbol esters in many cell types, suggesting a role for protein kinase C as a negative-feedback regulator of cell function [14–19]. In bovine chromaffin cells, Misbahuddin *et al.* [20] reported that Ca²⁺ mobilization induced by muscarinic-receptor stimulation was blocked by TPA treatment. The molecular mechanism underlying the inhibitory effect of TPA on the agonist-induced Ca²⁺ mobilization has not been defined.

In this study, we show that the PGE₂-induced phosphoinositide metabolism and Ca²⁺ mobilization in bovine chromaffin cells are inhibited by TPA treatment.

Abbreviations used: PGE₂, prostaglandin E₂; G-protein, GTP-binding protein; [Ca²⁺]_i, cytosolic free Ca²⁺ concentration; InsP₃, inositol trisphosphate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; GTP[S], guanosine 5'-[γ-thio]triphosphate; InsP₁, inositol monophosphate; InsP₂, inositol bisphosphate; G_i, G-protein that mediates inhibition of adenylate cyclase; G_o, a similar GTP-binding protein of unknown function.

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There are several points at which the agonist-induced phosphoinositide metabolism could be modulated by protein kinase C: the receptor, G-protein, or phospholipase C, with possible alteration of receptor-mediated activation of G-protein, or of G-protein-mediated activation of the phospholipase C. In this report, we have demonstrated that the inhibitory action of TPA is specific for the receptor-linked phosphoinositide metabolism and may occur at a stage distal to the coupling of receptors to G-proteins.

EXPERIMENTAL

Materials

myo-[2-³H]Inositol (20.0 Ci/mmol) was purchased from Amersham. [5,6,8,11,12,14,15-³H(n)]PGE₂ (160 Ci/mmol) was purchased from Du Pont-New England Nuclear. Unlabelled PGE₂ was generously given by Ono Pharmaceuticals, Osaka, Japan. TPA and 4 α -phorbol 12,13-didecanoate were obtained from Funakoshi Pharmaceuticals (Tokyo, Japan); GTP[S] was from Boehringer Mannheim; 2,3-diphosphoglycerate was from Sigma; ionomycin was from Calbiochem-Behring. All other chemicals were of reagent grade.

Cell culture and measurement of [Ca²⁺]_i

Chromaffin cells were prepared from bovine adrenal medulla as described previously [21]. Cells were cultured for 3–4 days before use in Ham's F-10 medium supplemented with 10% (v/v) heat-inactivated fetal-calf serum. [Ca²⁺]_i was measured as described previously [4].

Phosphoinositide metabolism in intact cells and membranes

Chromaffin cells cultured in 35 mm-diam. dishes (3 × 10⁶ cells/dish) precoated with poly-L-lysine were labelled with [³H]inositol (1.5–3 μ Ci/ml) for 60 h. Cells were then washed with Hepes-buffered solution containing 115 mM-NaCl, 10 mM-LiCl, 4.7 mM-KCl, 2.2 mM-CaCl₂, 1.2 mM-MgCl₂, 1.2 mM-KH₂PO₄, 11 mM-glucose, 15 mM-NaHCO₃ and 15 mM-Hepes (pH 7.4) and pre-incubated with or without phorbol esters for the indicated times at 37 °C. Reactions were started by the addition of test agents. To terminate the reactions, the medium was quickly aspirated and 5% (w/v) trichloroacetic acid was added to each well. [³H]inositol phosphates were then extracted and separated by Bio-Rad AG1-X8 chromatography essentially as described by Berridge *et al.* [22].

In the membrane studies, chromaffin cells labelled with [³H]inositol were treated with or without 1 μ M-TPA for 10 min at 37 °C and then lysed in 20 mM-Tris/HCl (pH 7.4) containing 0.5 mM-EGTA and 0.5 mM-EDTA at 4 °C. Cells were scraped from the culture dishes and homogenized with a Dounce homogenizer (40 strokes). The homogenates were centrifuged at 100 *g* for 5 min, after which the supernatant was removed and further centrifuged at 30000 *g* for 15 min. The pellet was washed with an incubation buffer composed of 50 mM-Mes, 10 mM-LiCl, 5 mM-MgCl₂ and 3 mM-EGTA (pH 6.6) and centrifuged at 30000 *g* for 15 min. The pellet was resuspended in an incubation buffer to a protein concentration of 2.5–3.0 mg/ml. These membranes were used without further purification. Reactions were started by the addition of 200 μ l of membrane fractions to 200 μ l of incubation buffer containing 2 mM-2,3-di-

phosphoglycerate to inhibit the degradation of InsP₃ [23]. After incubation for 10 min at 37 °C, 600 μ l of cold 10% trichloroacetic acid was added to terminate the reaction.

Protein concentrations were determined by the method of Lowry *et al.* [24], with bovine serum albumin as a standard.

[³H]PGE₂ binding assay

[³H]PGE₂ binding to intact cells was conducted at 37 °C in Hepes-buffered solution (pH 7.4). Chromaffin cells (1.5 × 10⁶) were treated for 10 min with or without 0.3 μ M-TPA, and then 5 nM-[³H]PGE₂ (176000 d.p.m.) was added. After further incubation for 60 min, bound [³H]PGE₂ was separated from free [³H]PGE₂ by rapid filtration through a Whatman GF/B glass filter (diam. 2.4 cm). Non-specific binding was determined by using a 1000-fold excess of unlabelled PGE₂ in the incubation mixture. The specific binding was calculated by subtracting the non-specific binding from the total binding.

In the membrane studies, cells pretreated with 1 μ M-TPA for 10 min were homogenized and centrifuged to obtain membrane fractions as described above, except that the final suspension buffer consisted of 10 mM-potassium phosphate (pH 6.0), 1 mM-EDTA and 0.1 M-NaCl. [³H]PGE₂ binding to these membrane fractions was done in a similar manner as with intact cells.

RESULTS

Effects of phorbol esters on phosphoinositide metabolism and Ca²⁺ mobilization in bovine chromaffin cells

In a recent paper [4], we demonstrated that a 2 min incubation of 1 μ M-PGE₂ enhanced phosphoinositide metabolism in bovine adrenal chromaffin cells with a resultant increase in all three inositol phosphates, InsP₁ (+40%), InsP₂ (+80%) and InsP₃ (+40%). As shown in Fig. 1(a), the PGE₂-induced accumulation of [³H]-inositol phosphates was blocked by pretreatment for 10 min with TPA in a dose-dependent manner. Half-maximal inhibition was observed at around 20 nM. The dose-response curve agrees with that of TPA for the activation of protein kinase C in platelets [13] and that for the stimulation of catecholamine release from chromaffin cells [4]. TPA treatment slightly decreased the basal contents of [³H]inositol phosphates. 4 α -Phorbol 12,13-didecanoate, known to be inactive for protein kinase C, affected neither the basal content nor the PGE₂-induced formation of [³H]inositol phosphates at any concentration (Fig. 1a). Inhibition of the PGE₂-induced formation of [³H]inositol phosphates was observed within 1 min and was complete by 10 min after addition of 1 μ M-TPA (Fig. 1b).

Phosphoinositide metabolism has been shown to be followed by Ca²⁺ mobilization from the endoplasmic reticulum. Fig. 2 depicts representative tracings of fluorescence changes in suspensions of quin2-loaded chromaffin cells. In untreated cells, 1 μ M-PGE₂ increased [Ca²⁺]_i from the basal value of 130 nM to a maximum of about 400 nM. Although TPA had no effect on the basal [Ca²⁺]_i, pretreatment for 5 min with 1 μ M-TPA completely blocked the PGE₂-evoked Ca²⁺ mobilization. A rise in [Ca²⁺]_i induced by muscarine was also blocked by TPA treatment, as reported previously [20]. The inactive 4 α -phorbol 12,13-didecanoate was again without effect (results not shown). In contrast with its inhibition of

Fig. 1. Effect of phorbol ester on PGE₂-induced phosphoinositide metabolism

(a) Dose-dependency: chromaffin cells (3×10^6 cells/well) labelled with $[^3\text{H}]$ inositol ($1.5\text{--}3.0 \mu\text{Ci/ml}$) were washed, treated for 10 min with various concentrations of TPA (○, ●) or 4α -phorbol 12,13-didecanoate (△, ▲) in the presence of 10 mM LiCl, and then exposed to vehicle (○, △) or $1 \mu\text{M}$ -PGE₂ (●, ▲) for 2 min in the continuous presence of phorbol ester. The values (means $\pm$ S.E.M., $n = 3$) shown were calculated by the summation of $[^3\text{H}]$ -inositol phosphates ($\text{InsP}_1 + \text{InsP}_2 + \text{InsP}_3$). (b) Time course: cells were incubated at 37°C for indicated times with $1 \mu\text{M}$ -TPA and then stimulated with $1 \mu\text{M}$ -PGE₂. The values (mean $\pm$ S.E.M., $n = 3$) are expressed as percentages of the control.

receptor-mediated response, TPA had little or no influence on the increase in $[\text{Ca}^{2+}]_i$ induced by the Ca^{2+} ionophore ionomycin.

Effect of TPA on specific $[^3\text{H}]$ PGE₂ binding to intact cells and membrane fractions

To elucidate the site of action of TPA, we first examined the possible effect of TPA on the binding of $[^3\text{H}]$ PGE₂ to intact cells. Chromaffin cells were pretreated with $0.3 \mu\text{M}$ -TPA for 10 min and then incubated with 5 nM - $[^3\text{H}]$ PGE₂ in the continuous presence of TPA. As shown in Table 1, specific $[^3\text{H}]$ PGE₂ binding to untreated and TPA-treated cells was 24 ± 0.9 and $22 \pm 0.7 \text{ fmol}/10^6$ cells respectively, suggesting that the number of high-affinity binding sites is only nominally affected by treatment of the cells with TPA.

We recently reported that the PGE receptor in bovine adrenal medulla is coupled to a G-protein and that specific $[^3\text{H}]$ PGE₂ binding to plasma membranes is decreased by the addition of guanine nucleotides, presumably owing to conversion of the receptor into the low-affinity state [3]. So we next investigated whether TPA treatment affected GTP-sensitivity of $[^3\text{H}]$ PGE₂ binding to receptors in membrane fractions. In control membranes, specific $[^3\text{H}]$ PGE₂ binding was decreased to 76 ± 2.2 from $125 \pm 9.6 \text{ fmol/mg}$ of protein by addition of $100 \mu\text{M}$ -GTP[S] (Table 1). However, the ability of GTP[S] to decrease binding was also observed in membranes prepared from TPA-treated cells (69 ± 3.7 from $110 \pm 5.2 \text{ fmol/mg}$ of protein). These results suggest that the site of action of TPA is distal to the PGE receptor and its coupling to a G-protein.

Effect of TPA on phospholipase C activity in membranes and chromaffin cells

Numerous investigators have recently suggested that the phospholipase C responsible for agonist-induced phosphoinositide metabolism is associated with cellular membranes and regulated by a G-protein [5–8]. In membranes prepared from $[^3\text{H}]$ inositol-labelled chromaffin cells, the formation of $[^3\text{H}]$ inositol phosphates was minimal at a physiological concentration of Ca^{2+} ($0.1 \mu\text{M}$), but it was enhanced synergistically with $0.3 \mu\text{M}$ free Ca^{2+} and $1 \mu\text{M}$ -GTP[S] (Table 2). Phospholipase C activity was also directly activated by a high concentration of Ca^{2+} ($10 \mu\text{M}$) only. These results indicate that membrane preparations from chromaffin cells contain guanine-nucleotide-stimulated phospholipase C, which requires Ca^{2+} for activity. However, the formation of $[^3\text{H}]$ InsP₂ and $[^3\text{H}]$ InsP₃ in membranes was not potentiated by PGE₂ above that generated in the presence of GTP[S] and Ca^{2+} , suggesting that phospholipase C or other factor(s) responsible for the PGE₂-induced formation of inositol phosphates might be lost or degraded during isolation of membranes. Therefore we investigated the effect of TPA on the phospholipase C activity, using intact chromaffin cells.

Muscarine, like PGE₂, is known to stimulate the formation of inositol phosphates through its specific receptor in bovine chromaffin cells [25]. On the other hand, AlF_4^- has been demonstrated to induce inositol phosphate formation in hepatocytes [26], neutrophils [27] and CCL39 cells [28] through a direct activation of the G-protein for its coupling to phospholipase C. In chromaffin cells, addition of 10 mM -NaF and $10 \mu\text{M}$ - AlCl_3 was also found to induce the formation of inositol

Fig. 2. Effect of TPA on PGE₂- and ionomycin-induced Ca²⁺ mobilization

Cells were loaded with quin2, washed, and resuspended in Hepes-buffered solution. Resuspended cells (3×10^6 cells/assay) were preincubated for 5 min with vehicle or $1 \mu\text{M}$ -TPA and then stimulated with $1 \mu\text{M}$ -PGE₂ and $2 \mu\text{M}$ -ionomycin at the points marked by arrows. Calibration of $[\text{Ca}^{2+}]_i$ is indicated at the left.

Table 1. Effect of TPA on specific [³H]PGE₂ binding to intact cells and membrane fractions

Chromaffin cells were treated with or without $0.3 \mu\text{M}$ -TPA for 10 min and then incubated with 5 nM -[³H]PGE₂. Membrane fractions were prepared from cells pretreated with or without $1 \mu\text{M}$ -TPA and incubated with 5 nM -[³H]-PGE₂ in the presence or absence of $100 \mu\text{M}$ -GTP[S]. All values were corrected for non-specific binding as described in the Experimental section and represent means $\pm$ S.E.M. ($n = 3$).

Treatment	Specific [³ H]PGE ₂ binding		
	Intact cells (fmol/ 10^6 cells)	Membrane fractions (fmol/mg of protein)	
		No GTP[S]	+ GTP[S] ($100 \mu\text{M}$)
Control	24 ± 0.9	125 ± 9.6	76 ± 2.2
TPA	22 ± 0.7	110 ± 5.2	69 ± 3.7

Table 2. Effect of Ca²⁺, GTP[S] and PGE₂ on the phospholipase C activities in membranes

Membranes prepared from [³H]inositol-labelled chromaffin cells were incubated for 10 min in the presence of 1 mM -2,3-diphosphoglycerate with the indicated additions. The values (means $\pm$ S.E.M., $n = 3$) shown were corrected by subtracting the basal amounts of [³H]inositol phosphates.

Additions	[³ H]Inositol phosphates (d.p.m./mg of protein)		
	InsP ₁	InsP ₂	InsP ₃
None	276 ± 19	1439 ± 32	173 ± 22
$0.3 \mu\text{M}$ -Ca ²⁺	383 ± 38	1881 ± 102	224 ± 1
+ $1 \mu\text{M}$ -GTP[S]	670 ± 55	2239 ± 61	338 ± 13
+ $1 \mu\text{M}$ -GTP[S]	759 ± 25	2168 ± 24	318 ± 4
+ $1 \mu\text{M}$ -PGE ₂			
$10 \mu\text{M}$ -Ca ²⁺	891 ± 15	3528 ± 28	780 ± 44

phosphates in the presence of LiCl, and their accumulation proceeded linearly over 30 min (results not shown). Furthermore, two groups have recently demonstrated that a rise in $[\text{Ca}^{2+}]_i$ could activate phospholipase C directly in chromaffin cells [9,10]. So we compared the effect of TPA on the activation of phospholipase C induced by PGE₂, muscarine, NaF plus AlCl₃, and the Ca²⁺ ionophore ionomycin. As shown in Table 3, the formation of [³H]inositol phosphates induced by muscarine was inhibited by TPA pretreatment as was that induced by PGE₂. However, formation induced by NaF plus AlCl₃ and by ionomycin was not affected by TPA, indicating that TPA did not affect the phospholipase C activity and the ability of G-protein to activate phospholipase C in the whole cells. Consistent with Ca²⁺ mobilization shown in Fig. 2, the inhibitory effect of TPA was specific for the receptor-mediated phosphoinositide metabolism.

DISCUSSION

In this study, we demonstrated that TPA inhibits PGE₂-induced phosphoinositide metabolism and Ca²⁺ mobilization in bovine adrenal chromaffin cells. These effects of TPA may be mediated through activation of protein kinase C, because of the ineffectiveness of the inactive phorbol ester 4 α -phorbol 12,13-didecanoate and because of the necessity for preincubation (Fig. 1). We demonstrated that PGE₂ might enhance catecholamine release from chromaffin cells in the presence of ouabain by activating protein kinase C [4]. By contrast with a rapid and transient release caused by nicotine-induced membrane depolarization, the release of catecholamines induced by PGE₂ and ouabain was gradual and sustained. These observations show the dual action of protein kinase C: negative-feedback control and positive forward or sustained control of cellular functions, depending on

Table 3. Effect of TPA on PGE₂-, muscarine-, ionomycin-, or NaF + AlCl₃-induced formation of inositol phosphates

[³H]inositol-labelled chromaffin cells (3×10^6 cells/assay) were pretreated with or without 1 μ M-TPA for 10 min, and then stimulated with 1 μ M-PGE₂, 100 μ M-muscarine or 1 μ M-ionomycin for 2 min, or with NaF + AlCl₃ for 10 min. When NaF + AlCl₃ was used as the stimulant, the concentration of Ca²⁺ in the Hepes-buffered solution was lowered to 1.0 mM. The values shown are means $\pm$ S.E.M. ($n = 3$).

Stimulant	TPA	[³ H]inositol phosphate (% of control)		
		InsP ₁	InsP ₂	InsP ₃
PGE ₂	—	122.0 $\pm$ 3.1	154.3 $\pm$ 1.5	117.0 $\pm$ 6.2
	+	98.8 $\pm$ 1.4	101.1 $\pm$ 2.0	100.9 $\pm$ 3.2
Muscarine	—	118.6 $\pm$ 3.6	141.2 $\pm$ 5.8	134.0 $\pm$ 9.6
	+	97.4 $\pm$ 1.7	104.9 $\pm$ 1.0	104.0 $\pm$ 1.7
Ionomycin	—	140.7 $\pm$ 5.9	147.3 $\pm$ 11.8	176.3 $\pm$ 21.2
	+	130.0 $\pm$ 2.2	148.8 $\pm$ 2.8	167.9 $\pm$ 1.6
NaF + AlCl ₃	—	158.1 $\pm$ 2.3	177.3 $\pm$ 5.0	140.2 $\pm$ 6.4
	+	148.6 $\pm$ 1.8	180.7 $\pm$ 3.9	152.2 $\pm$ 3.1

the functional consequence of the phosphorylation of each substrate protein. After TPA treatment, the G-protein demonstrated normal association with the PGE receptor, as determined by high-affinity binding to [³H]-PGE₂ and GTP[S]-sensitivity (Table 1), indicating that the inhibitory effect of TPA occurs at a stage distal to the coupling of the receptor to G-protein. The ability of TPA to block the muscarine-induced [Ca²⁺]_i rise and [³H]-inositol phosphate formation (Table 3) also suggests that the effect of phorbol ester is on a more general component(s) of phosphoinositide metabolism rather than a specific receptor.

Chromaffin cells contain a membrane-associated phospholipase C which is activated by GTP[S] in the presence of a low dose of Ca²⁺ or by a high concentration of Ca²⁺ alone. This phospholipase C activity was neither stimulated by PGE₂ (Table 2) nor inhibited by TPA (results not shown). In the present study, therefore, we used intact chromaffin cells and analysed the individual steps in the receptor-G-protein-phospholipase C transduction mechanism by stimulation of the cells with three different agents: agonist PGE₂ or muscarine, NaF plus AlCl₃, and ionomycin. We first demonstrated in chromaffin cells that treatment of the cells with NaF plus AlCl₃ resulted in the production of inositol phosphates (Table 3). Although we did not directly demonstrate the involvement of a G-protein in these cells, the recent demonstration of a transducing G-protein coupling the hormonal receptor to the activation of phospholipase C and of its stimulation by AlF₄⁻ in other cells makes a G-protein(s) a possible target for the AlF₄⁻ effects that we observed. It was demonstrated in whole cells, as well as in membranes, that the regulatory mechanism for the formation of inositol phosphates is not identical for the receptor-mediated and the AlF₄⁻ or ionomycin-induced pathways. That is, the receptor-induced Ca²⁺ mobilization and formation of inositol phosphates were completely blocked by TPA, whereas phosphoinositide metabolism stimulated by ionomycin and NaF plus AlCl₃ was not affected by TPA (Fig. 2 and Table 3). These results

indicate that phospholipase C itself and the coupling of the activated G-protein to phospholipase C are intact in the whole cells as well as in membranes, and that the inhibitory effect of TPA is on a component(s) specific to the agonist-induced phosphoinositide metabolism. Pfeilschifter & Bauer [29] have recently reported that TPA treatment attenuates the angiotensin II-induced, but not GTP[S]-induced, formation of inositol phosphates in mesangial-cell membranes. Therefore, it is conceivable that a G-protein coupling the receptor to phospholipase C may be a target for protein kinase C. In fact, it was reported that protein kinase C was able to phosphorylate an α subunit of G_i, thereby suppressing its function [30].

We recently reported the purification to apparent homogeneity of a pertussis-toxin-sensitive G-protein with a 40 kDa α -subunit, different from G_i and G_o, and suggested it to be the major G-protein in adrenal medullary membranes [31]. However, this G-protein is not coupled to the PGE receptor in the membrane, because the PGE₂-induced phosphoinositide metabolism and [Ca²⁺]_i rise were not prevented by pretreatment with pertussis toxin (H. Yokohama, unpublished work), consistent with our previous report that the G-protein coupled to the PGE receptor is insensitive to pertussis toxin [3]. Thus the G-protein which is coupled to the PGE receptor and to phospholipase C remains to be shown in bovine adrenal medulla.

Although it is now well established that mobilization of Ca²⁺ from its intracellular stores after receptor stimulation is the consequence of InsP₃ formation [12], a rise in [Ca²⁺]_i by ionomycin triggered the resultant formation of inositol phosphates in chromaffin cells ([9,10]; Table 3). Although the PGE receptor is thought to be linked to phospholipase C by a G-protein in chromaffin cells, Ca²⁺ mobilization and accumulation of inositol phosphates were greatly decreased by removal of extracellular Ca²⁺ (H. Yokohama, unpublished work). Similar results were reported with the muscarinic receptor [9]. Therefore it is possible that phospholipase C with low sensitivity to Ca²⁺, which is activated by massive Ca²⁺ entry from the extracellular medium through Ca²⁺ channels in plasma membranes or via another route, is involved in the initiation and/or amplification of receptor-mediated inositol phosphate formation. Recent reports have suggested that a G-protein may serve as a transducer for the coupling of the receptor to the Ca²⁺ channel [32,33]. Whatever the cause of stimulatory effects of PGE₂, the results of our present study suggest that a G-protein coupled to receptors or an unidentified factor(s) can be a target for protein kinase C and may lose the ability to transfer the signal from the receptor to phospholipase C properly. In the near future, the characterization of a G-protein and/or phospholipase C which are involved in agonist-stimulated phosphoinositide metabolism in chromaffin cells may reveal the signal-transduction mechanism of PGE₂ and will resolve the exact action of phorbol ester.

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