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PHORBOL ESTERS AND THYROLIBERIN HAVE DISTINCT ACTIONS REGARDING STIMULATION OF PROLACTIN SECRETION AND ACTIVATION OF ADENYLATE CYCLASE IN RAT PITUITARY TUMOUR CELLS (GH_4C_1 CELLS)

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Abstract—The phorbol ester 12-O-tetradecanoyl phorbol 13-acetate (TPA) enhances the effects of TRH on phase II of prolactin secretion as well as on hormone synthesis at both low and high TPA receptor occupancy. Furthermore TPA, but not the biologically inactive substance 4α-phorbol 12,13-didecanoate (4α-PDD), stimulates the particulate bound adenylate cyclase with a time course paralleling that of TRH activation. However, the combined additions of TRH and TPA activate this cyclase in an additive manner while the Gpp(NH)p- and the forskolin-sensitive enzyme are unaffected by TPA addition. Polymyxin B, which inhibits protein kinase C, abolishes activation of adenylate cyclase by TPA without interfering with the stimulatory action of TRH. Also, when phosphatase activity is preferentially inhibited by pretreatment of the cells with sodium vanadate, the TRH-sensitive cyclase is unaltered, while TPA activation is obliterated. Maximal stimulation of adenylate cyclase by cholera toxin pretreatment, obliterated the actions of TRH and TPA. Cells pretreated with pertussis toxin retained their TRH-sensitive cyclase, however, TPA-responsiveness was lost.

We therefore suggest that the action of TPA as it relates to activation of adenylate cyclase, is probably mediated via the G_i component of the adenylate cyclase complex, while TRH stimulates the enzyme via the classical pathway involving the stimulatory GTP binding protein (G_s).

In the GH_4C_1 rat pituitary cell line, which produces and spontaneously secretes prolactin, both thyroliberin (TRH) and the phorbol ester 12-O-tetradecanoyl phorbol 13-acetate (TPA) stimulate prolactin secretion and synthesis [1]. In these cells TRH, within seconds, elevates the cytosolic calcium concentration ($[Ca^{2+}]_i$) probably due to D-inositol-1,4,5-trisphosphate ($InsP_3$) formation after hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5P_2) [2–5]. $InsP_3$ serves as a general second messenger by increasing $[Ca^{2+}]_i$ through release from intracellular stores [6], and is considered responsible for the initial TRH secretory response (phase I) which lasts less than 1 min. After phase I, the rate of prolactin secretion is reduced, but maintained at about 50 per cent of maximum rate for several min (phase II) [7–11]. Direct measurements of $[Ca^{2+}]_i$ using the Quin-2 method have demonstrated a similar biphasic pattern parallelling the two phases of hormone release [12, 13]. The rapid elevation of $InsP_3$ quickly subsides and can therefore not explain the sustained elevation and second rise of $[Ca^{2+}]_i$ after TRH treatment [2, 3, 5]. During the secretory phase I, Ca^{2+}-activated K^+-channels are opened due to elevation of $[Ca^{2+}]_i$ causing membrane hyperpolarization, increased resistance, and suppressed firing of partly Ca^{2+}-dependent action potentials [14, 15]. The spontaneous firing activity is regained after about 1 min of TRH exposure corresponding to the secretory phase II [16–18]. During this phase, an entry of extracellular Ca^{2+} occurs, and this is a prerequisite for full expression of the TRH action [12].

The other product of PtdIns-4,5P_2 hydrolysis is 1,2-diacylglycerol (DG), which is the assumed endogenous activator of protein kinase C. Different phorbol esters, such as TPA, affect cellular processes by mimicking the effect of DG on protein kinase C [19, 20]. TPA induces a rapid and small rise in $[Ca^{2+}]_i$ followed by an inhibitory phase with transiently lowered $[Ca^{2+}]_i$ finally leading into a progressive and sustained elevation. This late elevation of $[Ca^{2+}]_i$ lasts for several min in GH_4C_1 cells. Furthermore, TPA gives rise to a phase II type of secretion [10, 21]. Also the stimulation of adenylate cyclase activity in GH_4C_1 cells by vasoactive intestinal polypeptide (VIP) elicits a phase II type of prolactin secretion and elevates $[Ca^{2+}]_i$ with a time-course corresponding to their secretory responses [22, 23]. In the present work on GH_4C_1 cells, we show that both TRH and TPA stimulate the particulate bound cyclase, but that their molecular actions are different. Moreover, their effects on prolactin secretion and production are additive. This study opens the possibility that

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cAMP may be a mediator of the TRH induced phase II secretion and also plays a role in the action of TPA.

MATERIALS AND METHODS

Materials. Ham's F-10 medium, horse serum, calf serum and amphotericin B were purchased from Flow Laboratory (Ervine, U.K.). Penicillin and streptomycin were from Gibco, Europe (Glasgow, U.K.). The phorbol ester (12-O-tetradecanoyl phorbol 13-acetate (TPA)) was from Sigma Chemical Company (St Louis, MO) and TRH was purchased from F. Hoffman La-Roche AG (Basle, Switzerland). Creatine phosphate and creatine kinase were obtained from Calbiochem AG (Basle, Switzerland). Myokinase, polymyxin B, ATP, cAMP and GTP were purchased from Sigma (U.S.A.). Gpp(NH)p (guanyl 5'-yl-imidodiphosphate) was obtained from Boehringer Mannheim (Germany). [α-^{32}P]-ATP (20–40 Ci/mmol) and [^{3}H]-cAMP (25 Ci/mmol) were purchased from Amersham Co. (U.K.). Bordella pertussis toxin was obtained from List Biochemical Laboratories (U.S.A.). All chemicals were of analytic grade.

Cell culture experiments. The GH_4C_1 cell strain was grown in plastic tissue culture dishes, containing serum supplemented medium at 37° in a humidified atmosphere of 95% O_2 and 5% CO_2 as described previously [19]. Penicillin (50 IU/ml), streptomycin (50 μg/ml) and amphotericin B (2.5 μg/ml) were all added to the culture medium, which was changed every 2–3 days and always 24 hr before the experiment took place. At the time of the experiment, the monolayer cultures received new medium containing the additions as indicated for each experiment, and medium collected at the time intervals given for hormone measurements. Prolactin was measured in the medium as previously described and related to mg cell protein per dish [24].

Adenylyl cyclase assay. In brief, the enzyme assay was carried out in a final volume of 50 μl and 1 mM ATP (including 2.5×10^6 cpm/tube of [α-^{32}P]-ATP), 40 μM GTP, 1 mM cAMP (with 7800 cpm/tube of [^{3}H]-cAMP), 1.4 mM EDTA, 0.1 mM EGTA, 2.8 mM Mg^{2+}, 0.10 mM Ca^{2+} and 25 mM Tris–HCl (pH 7.4) together with a regenerating system for ATP consisting of 20 mM creatine phosphate, 0.2 mg/ml of creatine kinase and 0.02 mg/ml of myokinase [25]. TRH, TPA, Gpp(NH)p (guanyl 5'-yl imidodiphosphate) or forskolin was added just prior to the incubation, which was carried out at 35° for 20 min. The reactions were stopped with 0.1 ml of a "stopping solution" containing 10 mM cAMP, 40 mM ATP and 1% sodium dodecyl sulphate followed by mixing and immediate cooling to 0°. The [^{32}P]-cAMP formed and the [^{3}H]-cAMP added to monitor recovery were isolated using combined Dowex and alumina oxide chromatography. The overall recovery was 65–85 per cent and the reaction blanks were 3–6 cpm per 1×10^6 cpm of labelled ATP added. The final imidazole eluates were collected in scintillation vials containing 5 ml of Instagel (Packard) and radioactivity counted for 10 min in a liquid scintillation counter [25].

The results are given as pmoles cAMP produced per mg membrane protein per min and shown as mean values ± SD of triplicate determinations. All experiments were carried out twice (N = 6).

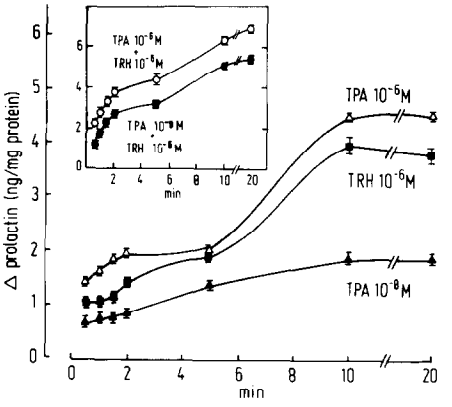

Fig. 1. The effects of TPA (10^{-8} M and 10^{-6} M) and TRH (10^{-6} M) separately and in combination on prolactin secretion from monolayer cultures of GH_4C_1 cells. The cells were treated for the indicated time periods and the first sampling period was after 30 sec. TRH at 10^{-6} M and TPA at 10^{-6} M elicited maximal secretory response, while TPA at 10^{-8} M gave 50% stimulation [1, 10]. The secretory effects of both high and low concentration of TPA were enhanced by the concomitant addition of TRH. The effect of 4α-phorbol 12,13-didecanoate (4α-PDD) was not different from controls (zero value). The data are shown as mean values ± SD from three different experiments (N = 9) where basal secretion at each point has been subtracted.

The effects of 4α-PDD were similar to basal activity (data not shown).

Statistical calculations. The results are given as mean values and statistical significance assessed by the Wilcoxon non-parametric, two-sample test.

RESULTS

Effects of TRH and TPA on prolactin secretion and production

Monolayer cultures of GH_4C_1 cells received TPA (10^{-8} M and 10^{-6} M) and TRH (10^{-6} M) alone or in combination. The first medium sample was collected after 0.5 min and the accumulation of prolactin during 20 min was followed and expressed as the difference between controls and treated cultures. The two concentrations of TPA were chosen since they gave half maximal and maximal effects, respectively [10, 21]. Using this experimental protocol the amount of prolactin released reflects the TRH induced phase II secretion, which TPA mimicks closely [10] (Fig. 1). When prolactin secretion was maximally stimulated by TRH (10^{-6} M), both concentrations of TPA enhanced the release in an additive manner (Fig. 1, inset).

Table 1 shows experiments using TRH and TPA in concentrations yielding maximal effects on prolactin production [20] during incubation periods of 24 hr and 72 hr, respectively. In monolayer cultures containing the same amount of cells, TRH and TPA stimulated hormone production confirming previous results [1, 20], and the combined treatment again resulted in additive effects. After cessation of treat-

Table 1. Effects of TRH and TPA on prolactin production in GH_4C_1 pituitary cells

Treatment groups	Prolactin production (μg/ml·24 hr) 24 hr	72 hr
Control	7.0 ± 0.5	8.2 ± 0.6
TRH(10^{-6} M)	15.3 ± 1.3*	18.5 ± 1.8*
TPA(10^{-6} M)	18.8 ± 1.9*	14.3 ± 1.1*
TPA(10^{-6} M) + TRH(10^{-6} M)	24.2 ± 1.1*†	33.6 ± 0.9*†

Monolayer cultures of GH_4C_1 cells in Ham's F-10 medium supplemented with 12 per cent serum received TRH and TPA as indicated for 24 and 72 hr. The medium was collected and prolactin measured radioimmunologically as described previously [24]. The production of prolactin, which is not degraded intracellularly or in the medium [37], was relatively constant during each 24-hr collection period. The results are shown as mean values and SD of triplicates from one experiment which was repeated twice (N = 6). Cellular protein concentration per dish was similar in the different groups (179 μg ± 15.1 (mean ± S.E.)). The combined action of TRH and TPA on prolactin production were significantly over and above the effects observed when only one of the substances was used.

* $P < 0.01$, treated groups vs controls.

† $P < 0.01$, combined treatment vs TRH or TPA treatment alone.

ment, there was a gradual and similar return to control values (data not shown).

Effects of TRH and TPA on particulate bound adenylate cyclase

There are indications that partly purified protein kinase C prevents inhibition of human platelet adenylate cyclase by epinephrine via inactivation of the G_i-component [26]. The GH_4C_1 cell has a hormone sensitive adenylate cyclase which is activated about 2-fold by TRH [25]. In the presence of a standard incubation mixture (low free [Mg^{2+}]), TRH and TPA in concentrations known to give maximum activation show a very similar time course and magnitude of cyclase activation (Fig. 2). In combination, the effects of the two substances on the cyclase were additive. In comparison, TPA is unable to change the rate of adenylate cyclase activation by the GTP analogue Gpp(NH)p and forskolin that act on G_s and on the catalytic site, respectively (Fig. 2, inset). Also, diacyl glycerol (e.g. OAG), an assumed activator of protein kinase C, is able to stimulate the cyclase (Table 2). The cyclase activations by TRH and TPA are similar with regard to GTP-dependency.

The following results suggest that G_i is inactivated by TPA: addition of polymyxin B (50 U/ml–100 U/ml), an inhibitor of protein kinase C [27], to the enzyme assay did not affect the basal activity of the cyclase, but already 50 U/ml abolished TPA activation (Table 3). However, the TRH-induced adenylate cyclase activation was only reduced about 11 per cent by the maximal concentration used. Also, adenylate cyclase activation by vasoactive intestinal polypeptide (VIP) was only reduced 14 per cent by polymyxin B when used in the maximal concentration. A further indication that the effect of TPA represented inactivation of the G_i-component of the GH_4C_1 cell cyclase was obtained by the TPA-induced blockade of the somatostatin (ST) effect. ST inhibits the cyclase through G_i activation (Table 4). Similar results were also obtained when measuring total cAMP accumulation in intact cells and PRL release (data not shown). When GH_4C_1 cells were pretreated with vanadate, causing a preferential

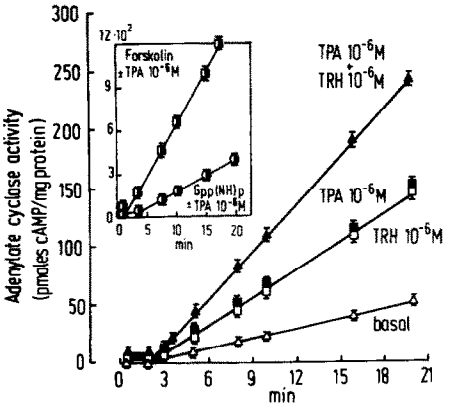

Fig. 2. The effects of TPA (10^{-6} M) alone and in combination with TRH (10^{-6} M), Gpp(NH)p (10^{-5} M) and forskolin (10^{-5} M) on the particulate bound adenylate cyclase in GH_4C_1 cells.

Table 2. The effects of 1-oleyl, 2-acetyl-glycerol (OAG) on the GH_4C_1 cell adenylate cyclase

Group	N	Formation of cAMP (pmoles/mg protein min)
Control	6	4.9 ± 0.1
OAG (10^{-7} M)	6	*5.6 ± 0.1
(10^{-6} M)	6	†7.8 ± 0.2

In the experiments testing the effect of OAG, phosphatidyl serine was not present in the incubation mixture which was otherwise as described in Materials and Methods using free [Mg^{2+}] of 0.4 mM and free [Ca^{2+}] of 0.1 mM. The results are shown as mean values ± SD of two separate experiments.

* $P < 0.05$.

† $P < 0.01$.

Table 3. The effect of polymyxin B on TRH- and TPA-stimulated adenylate cyclase in prolactin producing rat pituitary GH_4C_1 cells

Activating agent	Formation of cAMP (pmoles/mg protein · min) Polymyxin B concentration (U/ml) 0	50	100	1000
Control	5.1 ± 0.3	5.4 ± 0.5	5.7 ± 0.4	5.6 ± 0.2
TRH (10^{-6} M)	9.6 ± 0.6*	9.5 ± 0.3*	9.3 ± 0.5*	8.3 ± 0.9*
TPA (10^{-6} M)	10.2 ± 1.0*	5.3 ± 0.4	5.0 ± 0.2	5.2 ± 0.3

Monolayer cultures were treated with polymyxin B for 20 min as described in Materials and Methods. Prewashed cells were homogenized (Ultra-Turrax) in 10 volumes of Tris–HCl/EDTA buffer (10/1 mM, pH 7.4), and particulate membrane fraction prepared [24]. Twenty μl aliquots were added to an incubation mixture with a final free Mg^{2+} concentration of 0.4 mM in the absence or presence of TRH 10^{-6} M or TPA 10^{-6} M. The results are given as mean ± SD of triplicate determinations and expressed as pmoles cAMP produced per mg membrane protein per minute of incubation. The experiment was repeated three times with similar results (N = 9). Asterisks indicate significant differences ($P < 0.01$) to zero controls.

inhibition of phosphatase activity [28], the differential TRH-sensitive cyclase activity was unaltered while TPA-stimulation was obliterated (Table 5). Thus, TPA activates the cyclase in a way different from that of TRH. Interestingly, vanadate pretreatment of the cells stimulated the cyclase activity in a dose dependent manner, but the absolute effect contributed by TRH remained almost constant (Table 5). Whether the activation of basal enzyme activity by vanadate is only due to an altered state of G_i phosphorylation, is not known. However, an altered state of phosphorylation appears to be involved in the action of TPA on G_i.

To further examine the molecular mechanisms by which TRH and TPA activates the GH_4C_1 cell cyclase, experiments with cholera toxin and pertussis toxin that ADP-ribosylate G_s and G_i proteins of the adenylate cyclase complex, respectively, were performed. When membranes were pre-incubated with activated cholera toxin (10 μg/ml), a 20-fold increase in basal enzyme activity was demonstrated, and TRH and TPA were unable to further sustain the response (Table 6). Pretreatment of whole cells with pertussis toxin (10 μg/ml) increased basal enzyme activity 2.3-fold with full preservation of the TRH-sensitive cyclase, while the effect of TPA was obliterated (Table 6). These experiments further strengthened the suggestion that the TPA-dependent cyclase activation is mediated via G_i.

DISCUSSION

The phase II of prolactin secretion induced by TRH is mimicked by both TPA and vasoactive intestinal polypeptide (VIP) [10, 23, 29], and all three secretagogues elevate intracellular $[Ca^{2+}]$ [10, 22]. This study shows that both TRH and TPA activate the adenylate cyclase system, and that there exists an interconnection between this system and protein kinase C activation via G_i. The mechanism by which the cAMP system raises $[Ca^{2+}]_i$ is unknown, but both signal systems appear to be involved in prolactin secretion and production.

Phase II secretion in the action of TRH is dependent upon entrance of extracellular Ca^{2+} which is probably taken up via voltage dependent Ca^{2+}-channels during the generation of action potentials [30, 31]. Albert and Tashjian [21] have shown that the slow and sustained elevation of $[Ca^{2+}]_i$ after TPA treatment in GH_4C_1 cells is dependent on extracellular $[Ca^{2+}]$, and has a time course corresponding to stimulation of secretion. However, TPA regulates $[Ca^{2+}]_i$ in a biphasic manner which may be dependent on pre-existing $[Ca^{2+}]_i$ and whose molecular involvement and functional significance is not yet understood [21, 32]. Although there is a time-course resemblance of the effects of TPA with TRH on phase II secretion and prolactin synthesis, their effects are additive and their detailed molecular

Table 4. Inactivation of the TPA induced adenylate cyclase by somatostatin (ST)

Group	N	Formation of cAMP (pmoles/mg protein min)
Control	6	4.8 ± 0.1
TPA (10^{-6} M)	6	*9.6 ± 0.3
ST (10^{-5} M)	6	*3.0 ± 0.2
ST (10^{-5} M) + TPA (10^{-6} M)	6	5.2 ± 0.2

The enzyme was measured as described in Materials and Methods using free $[Mg^{2+}]$ and $[Ca^{2+}]$ of 0.4 mM and 0.1 mM, respectively. The results are shown as mean values ± SD of two separate experiments.

* $P < 0.05$.

Table 5. The effects of TRH and TPA on particulate bound adenylate cyclase activation in GH_4C_1 cells pretreated with sodium vanadate

Treatment group	Formation of cAMP (pmoles/mg protein · min) Sodium vanadate (μM) 0	20	40
Control	4.6 ± 0.3	6.2 ± 0.1	7.1 ± 0.4
TRH (10^{-6} M)	8.2 ± 0.6*	10.0 ± 1.1*	11.1 ± 0.5*
TPA (10^{-6} M)	8.6 ± 0.1*	6.8 ± 0.3	7.0 ± 0.6

Monolayer cultures were treated with sodium vanadate for 19 hr before harvest and preparation of the particulate membrane fraction [25]. The cyclase assay was performed with washed subcellular fractions in the absence of added vanadate as summarized in Materials and Methods. The results are shown as mean values ± SD of triplicates, and the experiment was repeated three times. Asterisks indicate results significantly ($P < 0.01$) different from control ($N = 9$).

Table 6. The influence of cholera toxin and pertussis toxin on basal, TRH- and TPA-activated adenylate cyclase of rat pituitary GH_4C_1 cells

Pretreatment groups	Treatment groups Basal	TRH	TPA
Control	4.9 ± 0.1	8.3 ± 0.5*	8.9 ± 1.0*
Cholera toxin	†101 ± 8.3	†103 ± 9.2	108 ± 9.6
Pertussis toxin	†11.5 ± 1.3	†19.6 ± 2.1	10.6 ± 0.7

The data represent formation of cAMP (pmoles/mg protein · min) in different groups

Membranes (pre-incubated with activated cholera toxin, 10 μg/ml) or whole cells (pretreated with pertussis toxin, 10 μg/l) were assayed for AC activity in the presence of 0.4 mM free Mg^{2+}, 1 mM ATP, 40 μM GTP and either TRH (10^{-6} M) or TPA (10^{-6} M). All values are expressed as means with SD of triplicate determinations. The experiment was repeated twice ($N = 6$).

* Indicates $P < 0.05$ for horizontal (within-group) comparisons with basal activity and † depicts $P < 0.05$ for vertical (between-group) comparisons with corresponding controls.

actions different as regards adenylate cyclase activation and $[Ca^{2+}]_i$ regulation [21, 33].

The detailed delineation of how the different TRH-evoked second messenger signals may operate in these cells to alter hormone secretion as well as gene expression, has proved intricate. However, this work shows that there may be an important cooperation between protein kinase C and adenylate cyclase. This possible interaction may prove important for mediating secretory phase II and stimulated hormone production in the TRH action, and it also makes possible feed-back mechanisms aimed to potentiate or to terminate the action of TRH on sites distal to ligand–receptor binding. An interaction between phorbol esters and cAMP in regulating ion transport has been described also for other biological systems. Phorbol esters inhibit K^+-currents evoked by cAMP in *Xenopus* oocytes [34]. Also, Baraban *et al.* [35] have shown that phorbol esters blocked the Ca^{2+}-dependent K^+-conductance in hippocampal pyramidal neurons, an effect similar to that previously observed following activation of cAMP dependent protein kinase [36]. Hence, this study also offers a possible explanation for these two apparently discrepant observations.

TPA has recently been shown to depolarize GH cells leading to increased firing of action potentials [10]. The present results open the possibility that TPA-induced inhibition of K^+-conductance [10] may occur via its effect on G_i.

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