Biochemical Pharmacology, Vol. 34, No. 21, pp. 3795–3802, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 +0.00

TETRADECANOYL PHORBOL-13-ACETATE COUNTERACTS THE RESPONSIVENESS OF CULTURED THYROID CELLS TO THYROTROPIN

BERNARD HAYE*, JEAN LOUIS AUBLIN, SERGE CHAMPION, BERNARD LAMBERT and CLAUDE JACQUEMIN

Laboratoire de Biochimie, U.E.R. des Sciences Exactes et Naturelles,
Boite postale 347 - 51062 Reims Cedex, France

(*Received* 25 *February* 1985; *accepted* 20 *May* 1985)

Abstract—We have studied the effects of TPA on the metabolism of porcine thyroid cells cultured for 1–4 days in the absence (control cells) and in the presence of 0.1 mU/ml TSH (TSH cells). The phospholipid turnover, evaluated after a 2 hr incorporation of ^{32}P-phosphate into phospholipids, is markedly modified by the presence of TPA (1.5 μM, 2 hr) in the incubation medium of control and TSH treated cells. The total incorporation is 3–4 times higher than untreated cells, the labelling of phosphatidylinositol (PI) is slightly decreased or unchanged whereas that of phosphatidylcholine (PC) is strongly increased. The increased labelling of PI, promoted by an acute TSH treatment is counteracted by TPA. This TPA effect is not observed when prelabelled cells are challenged for 5 min with the drug. A similar effect is observed when 10 nM TPA is added in the culture medium for 20 hr. The addition of TPA does not affect significantly the protein iodine content in 3 or 4 days control cells incubated for 45 min or 2 hr with ^{125}I-iodine, but dramatically decreases the very high iodination rate of TSH cells. We have tested the TPA effect on the cyclic AMP accumulation for the last 5 min of a 2 hr incubation. TPA inhibits by about 50–80% the stimulation evoked by TSH and only by 10% that evoked by forskolin (0.1 mM). These results suggest a possible link between the PC turnover and the adenylate cyclase responsiveness to TSH and the iodination rate.

The tumor promoting phorbol ester TPA† and its analogs induce various perturbations on biological and biochemical parameters in different cell types [1, 2]. TPA affects Ca^{2+} mobilization [3], cyclic nucleotide [4] and phospholipid metabolism [5–8], protein-kinase C activity [9–11], superoxide radicals production [12] and other events [13, 14]. The effect of tumor promoting phorbol esters on thyroid cells has not been described. As previously reported [15], the presence of 0.1 mU/ml TSH in culture medium allowed porcine thyroid cell reorganisation into a follicular like structure and a high adenylate-cyclase sensitivity to acute TSH stimulation (TSH cells). In contrast, when cultured in the absence of TSH, thyroid cells displayed inverted polarity and a low adenylate-cyclase sensitivity (control cells). In a previous work [16] we studied the chronic and acute effects of TSH on PI turnover in cultured porcine thyroid cells, in relation to the adenylate cyclase activity. We described a "chronic phospholipid effect" on cultured thyroid cells in the presence of 0.1 mU/ml TSH. This chronic effect of TSH (stimulation of ^{32}P-incorporation into PI) can be reproduced by a chronic treatment with dibutyryl cyclic AMP (10 mM) or prostaglandin E_2 (10 μM) which did not evoke the classical acute phospholipid effect in a 2 hr incubation. The stimulation of the ^{32}P-incorporation into PI is attributed to the activity of PI-specific phospholipase C. On the other hand a stimulation by TPA of a membrane associated phospholipase C which generates 1–2 diacylglycerol from PC was described in cultured myoblasts [8]. These preliminary experiments were done to evidence if TPA induced some perturbations on cultured thyroid cells metabolism. Three parameters representative of the activity of thyroid cells were followed: cyclic AMP accumulation, phospholipid metabolism and iodide organification.

Our results indicate that TPA:

(a) acutely stimulates the turnover of the phosphoryl moiety of phosphatidylcholine;

(b) uncouples adenylate cyclase from the TSH receptors;

(c) decreases the very high iodination rate of TSH cells.

MATERIALS AND METHODS

Preparation, culture and washing of thyroid cells. The preparation, the culture and washing of thyroid cells were as in [15]. Thyroid cells were isolated from pig glands by a discontinuous trypsinization technique. Freshly isolated cells, suspended in Eagle minimum essential medium, pH 7.4, with 10% (v/v) calf serum, penicillin (200 units/ml) and streptomycin sulfate (0.05 mg/ml) were incubated, at a concentration of 2 to 3 × 10^6 cells/ml in falcon plastic Petri dishes, untreated for tissue culture, at 37° in 95% air–5% CO_2 atmosphere with or without TSH.

* To whom all correspondence should be sent.

† Abbreviations used: TSH, thyrotropin; TPA, tetradecanoyl phorbol-13-acetate; hepes, N-2 hydroxyethylpiperazine-N′-2 ethane sulfonic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; EGF, epidermal growth factor.

At the conclusion of the culture period, cells were centrifuged at 200 *g* for 7 min. The pellet was resuspended on 25 mM Earle–Hepes, pH 7.2, buffer and the cells were centrifuged again, this washing procedure being repeated twice. After the last washing, the cells were suspended in 25 mM Earle–Hepes buffer, pH 7.2. The "acute treatment" was performed during the incubation of the cells after the washings, the maximal acute TSH effect was obtained between 10 and 40 mU/ml.

Cyclic AMP assay. Aliquots (0.4 ml, 0.1–0.3 mg protein) of washed thyroid cell suspension were preincubated from 0 to 120 min in the presence or absence of TPA (1.5 μM). Then the incubation was pursued 5 min with or without TSH (40 mU/ml) and with IBMX (1 mM). The incubation was terminated by the addition of 78 μl 10 N $HClO_4$ and immersion of the tubes into ice bath. The cells were homogenized and the cyclic AMP content was assayed by the radioimmunological method of Cailla *et al.* [17] except that bound and free ligand were separated by precipitation of bound ligand with a mixture of γ-globulin (2.5 mg/ml) in 0.1 M citrate buffer (pH 6.2) and polyethylene glycol 6000 (20 g/100 ml water).

Phospholipid assay. Aliquots (0.5 ml, 0.1–0.3 mg protein) of washed thyroid cell suspension were air-incubated for 2 hr at 37° in final volume of 0.7 ml containing 25 mM Earle–Hepes buffer (pH 7.2), ^{32}P-orthophosphate (10 μCi) and the effectors. The incubation was stopped by the addition of a chloroform–methanol mixture (2:1, v/v). The phospholipids were extracted and analysed as previously described [18]. After homogenization and centrifugation, the lower phase was concentrated and chromatographed according to Marinetti and Stotz [19]. The individual phospholipids were visualized by spraying the chromatogram with a solution of rhodamine 6G (0.15, w/v). The PI (cpm/mg protein)/PC (cpm/mg protein) ratio is calculated as an index of the "phospholipid effect".

Thyroid protein iodination ($PB^{125}I$). After being washed, the cells were incubated for 45 min at 37° in 25 mM Earle–Hepes buffer (pH 7.2) in 0.7 ml final volume containing 1 μCi Na ^{125}I. At the end of the incubation, 1.3 ml 25 mM Earle–Hepes buffer containing KI (0.1 mM) and bovine serum albumin fraction V (5 mg/ml), then 2 ml cold 20% (w/v) trichloracetic acid were added. After centrifugation (500 *g* for 5 min) the pellet was resuspended and washed with cold 10% trichloracetic acid (2 times). The pellet was counted as PB ^{125}I (protein bound iodine).

Other methods and chemicals. Protein estimation was done as in [20] using bovine serum albumin fraction V in 0.1 M NaOH as a standard. Aliquots of cells were centrifuged and the pellets were solubilized in 0.1 M NaOH. Bovine TSH 3.5 IU/mg (NIAMDD, bTSH B8) and ovine TSH 7.5 IU/mg (NIAMDD, oTSH 9) were a gift of the NIH (Bethesda MD). ^{32}P-orthophosphate (10 mCi/ml) and Na ^{125}I (2 mCi/ml) were purchased from the CEN Saclay. Trypsin was purchased from Gibco, new-born calf serum from Flow Lab. (Irvine), minimum essential medium from Merieux (Lyon), TPA, phorbol 13-acetate, forskolin from Sigma. Phorbol derivatives were dissolved in dimethylsulfoxide, the other assays were supplemented with the same solvent. Forskolin was dissolved in ethanol, the other assays were supplemented with the same solvent. Solvent concentration was no more than 0.1% (v/v).

Statistical evaluations. The statistical differences were calculated using Student's *t*-test; mean values of triplicate experiments were expressed with standard errors of the mean (S.E.M.). Experiments were reproduced 2–3 times with very similar or identical results. A P value below 0.01 was considered significant.

RESULTS

Effects of TPA on isolated thyroid cell morphology

In a preliminary work we tested the concentration response and incubation time effect of TPA on control and TSH treated cells. A rapid (1–2 hr) morphological and substratum-adherence change was observed under light microscopy in TPA treated thyroid cultured cells. The maximum acute effect was observed with 1.5 μM TPA for 1–2 hr. A similar effect was obtained with 10 nM TPA added for 20 hr in culture medium. Cell clusters cultured in dishes untreated for tissue culture, remained in suspension. When treated with TPA, some aggregates attached to the polystyrene surface within 12 hr, spread out and formed a monolayer after 20 hr. This monolayer did not display a mosaic-like structure as usually observed with a typical pig thyroid epithelial monolayer. Cells were strongly flattened and those situated at the periphery of the layer exhibited some processes. One day thereafter some cells were no longer adjacent to the monolayer; these cells presented a flat extended configuration and expressed long thin processes.

In subsequent experiments we used 1.5 μM TPA in acute effects on phospholipid metabolism, cyclic-AMP accumulation and protein iodination.

Effect of TPA on thyroid phospholipid metabolism

The ^{32}P incorporation into phospholipids was markedly modified by the presence of TPA (1.5 μM) in the incubation medium of control and of treated cells. On 1 day cultured cells, the ^{32}P incorporation into total phospholipids was respectively 11330 ± 410 cpm/mg protein without TPA and 33135 ± 520 cpm/mg protein with it ($P < 0.001$). Contrarily to TSH which specifically stimulated PI turnover, TPA strongly increased the ^{32}P incorporation into PC (Fig. 1). In the presence of TPA the labelling of PI and phosphatidic acid (not shown) were slightly decreased or unchanged. The increased labelling of PI promoted by an acute TSH treatment was counteracted by TPA and the increased labelling of PC promoted by an acute TPA treatment was partially counteracted by TSH. This TPA effect was not observed when ^{32}P labelled cells (2 hr) were challenged for 5 min with the drug (not shown).

On 4 day cultured cells (Fig. 2), comparable results were obtained. The ^{32}P incorporation into total phospholipids was respectively 6910 ± 270 cpm/mg protein without TPA and 23630 ± 710 cpm/mg protein with TPA ($P < 0.001$). The effect of TPA on PC turnover was again observed but the antagonism

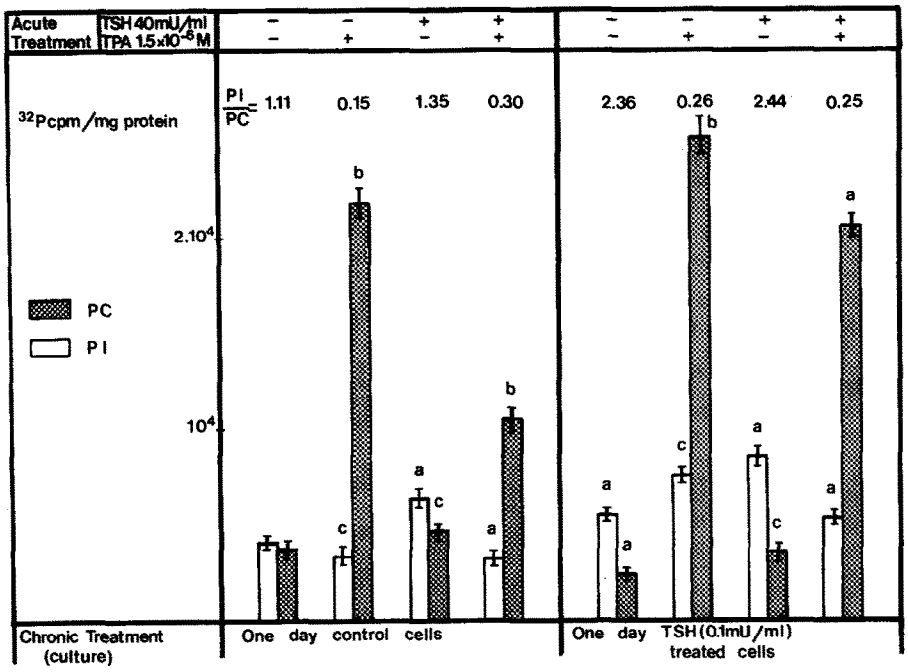

Fig. 1. Effect of TPA on the phospholipid metabolism of 1 day cultured pig thyroid cells. After washing, cells were labelled for 2 hr with ^{32}P-orthophosphate in the presence or absence of TSH (40 mU/ml) and/or TPA (1.5 μM). (a) $P < 0.01$. PI : control cells + TSH (acute) vs control cells; PI: control cells + TSH + TPA (acute) vs control cells + TSH (acute); PI: TSH cells vs control cells; PI: TSH cells + TSH (acute) vs TSH cells; PI: TSH cells + TSH and TPA (acute) vs TSH cells + TSH (acute); PC: TSH cells vs control cells; PC: TSH cells + TSH and TPA (acute) vs TSH cells + TPA (acute). (b) $P < 0.001$. PC: control cells + TPA (acute) vs control; PC: control cells + TSH and TPA (acute) vs control cells + TPA (acute); PC: control cells + TSH and TPA (acute) vs control cells; PC: TSH cells + TPA (acute) vs TSH cells. (c) Not statistically different from corresponding control.

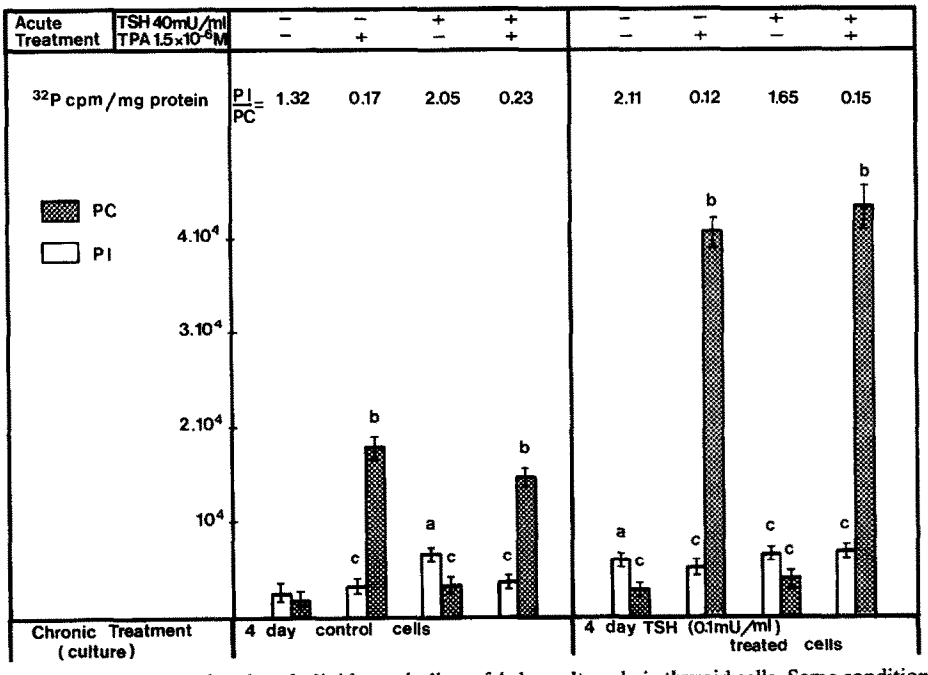

Fig 2. Effect of TPA on the phospholipid metabolism of 4-day cultured pig thyroid cells. Same conditions as in Figure 1. (a) $P < 0.01$. PI: control cells + TSH (acute) vs control cells; PI: TSH cells vs control cells. (b) $P < 0.001$. PC: control cells + TPA (acute) vs control cells; PC: control cells + TSH and TPA (acute) vs control cells and vs control cells + TSH (acute); PC: TSH cells + TPA (acute) vs TSH cells; PC: TSH cells + TSH and TPA (acute) vs TSH cells. (c) Not statistically different from corresponding control.

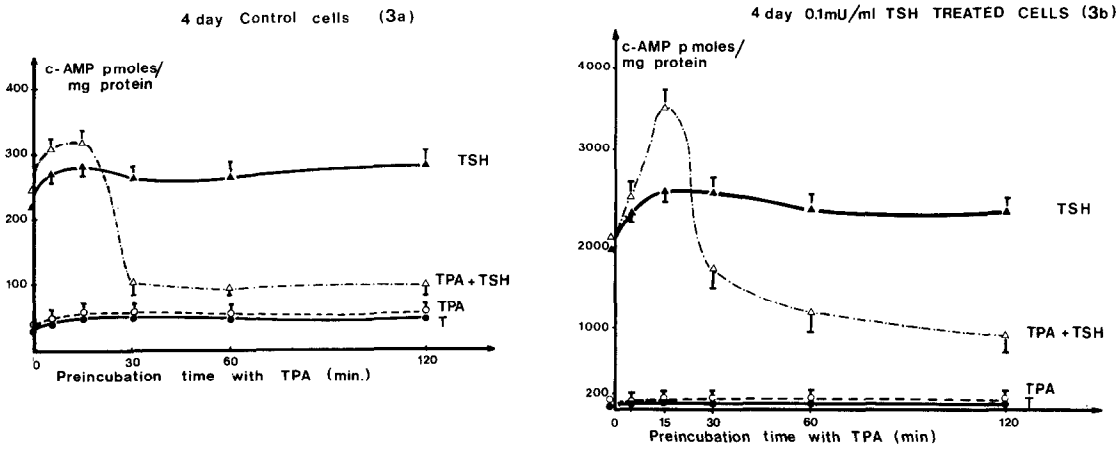

Fig. 3. Effect of the preincubation (0–2 hr) with TPA (1.5 μM) on the subsequent acute stimulation (5 min) with 40 mU/ml TSH, of the cyclic AMP accumulation by 4-day cultured cells. Cells were cultured 4 day without (control cells) or with TSH (0.1 mU/ml) (TSH-treated cells). After washings, cells were incubated from 0 to 120 min in the presence or absence of TPA (1.5 μM). Then the incubation was pursued 5 min with or without TSH (40 mU/ml) and IBMX (mM) and was terminated with $HClO_4$ 10 N. For the cAMP determination see Materials and Methods. The presented results were identical in three distinct experiments. (a) 4-day control cells; (b) 4-day TSH-treated cells. $P < 0.01$, TSH vs TSH + TPA at 15 min; $P < 0.001$, TSH vs TSH + TPA from 30 to 120 min.

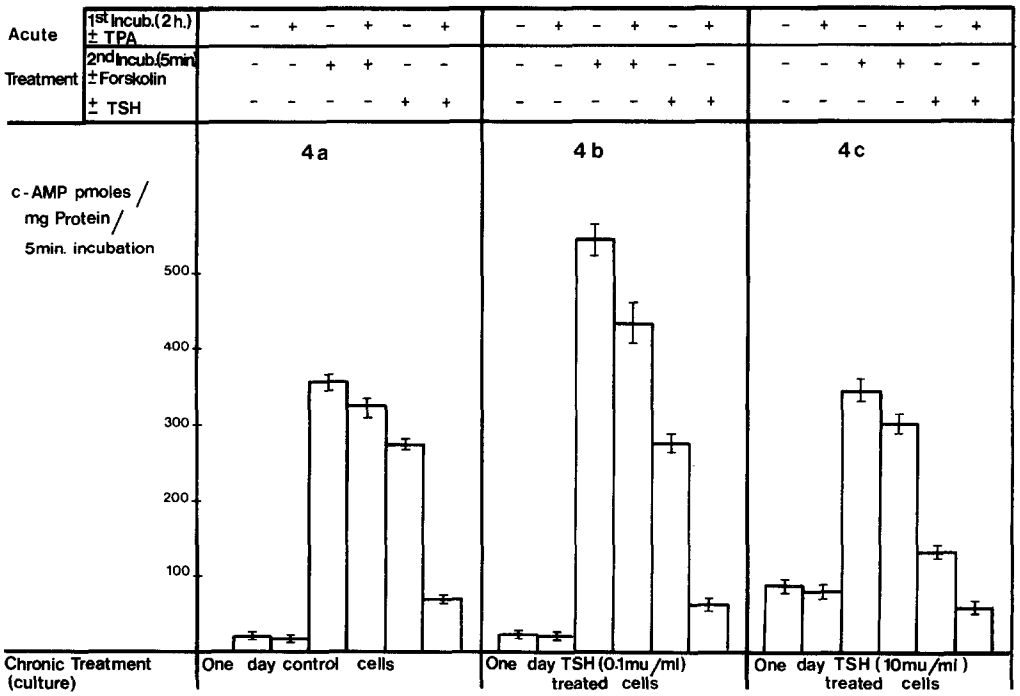

Fig. 4. Effect of preincubation with TPA (1.5 μM) on the cyclic AMP accumulation under acute subsequent incubation with TSH (40 mU/ml) or forskolin (0.1 mM) of 1-day cultured pig thyroid cells. After washings, cells were preincubated from 0 to 2 hr with TPA (1.5 μM) then the effectors TSH or forskolin were introduced with IBMX (mM) and the incubation was pursued for 5 min. The incubation was terminated with $HClO_4$ 10 N. See Materials and Methods for cyclic AMP determinations. (a, b) Not statistically significant: TPA vs control, TPA + forskolin vs forskolin; $P < 0.001$: forskolin vs control, TPA + forskolin vs TPA, TSH vs control, TPA + TSH vs TSH; $P < 0.01$: TSH + TPA vs TPA. (c) Not statistically significant: TPA vs control, TPA + TSH vs TPA, TPA + forskolin vs forskolin; $P < 0.001$: forskolin vs control, TPA + forskolin vs TPA; $P < 0.01$: TSH vs corresponding control.

between TPA and TSH effects was not statistically significant. The other phospholipids were unaffected by TPA treatment (not shown). The absolute amount of phosphatidylcholine was not modified by acute TPA treatment, only the specific radioactivity was increased.

Effect of TPA on the protein iodination

The addition of TPA (1.5 μM) in a 45 min incubation did not modify the iodinating capacity of 4 day cultured control cells (22100 ± 350 cmp/mg protein without TPA and 20500 ± 830 cpm/mg protein with TPA) but dramatically decreased the very high one of 4 day 0.1 mU/ml TSH treated cells (2,050,000 ± 15,000 cpm/mg protein without TPA and 210,000 ± 12,000 cpm/mg protein TPA) ($P < 0.001$).

Effect of TPA on cyclic AMP accumulation

The effects on cyclic AMP accumulation by cultured cells depended on the conditions of treatment. We studied the effects of the presence of TPA in the incubation medium of cells cultured from 1 to 4 days in the presence or absence of 0.1 mU/ml TSH, the TPA treatment (0–2 hr) being followed by an acute stimulation (5 min) with TSH (40 mU/ml) (Fig. 3). The results were similar on the control and TSH treated cells. The basal cyclic AMP levels of cells was not affected by their pre-treatment with TPA up to 2 hr. The 5 min stimulation by TSH increased 5- and 24-fold the cyclic AMP level in the control and TSH treated cells respectively. The presence of TPA during the preincubation had a biphasic effect: a transient increase of cyclic AMP accumulation obtained after 15 min was followed by a strong inhibition of the TSH effect after 30 min (Fig. 3a). The effects of the pretreatment with TPA were enhanced in cells cultured 4 days in the presence of 0.1 mU/ml TSH (Fig. 3b). In the next experiments, the effects of the treatment with TPA on the sensitivity to TSH or forskolin induced accumulation of cyclic AMP, were compared after 30 min or 2 hr incubation periods to fit in the inhibitory phase. On 1 day cultured cells (Fig. 4), preincubation with TPA for 2 hr strongly decreased the acute effect of 40 mU/ml TSH added for 5 min during an incubation of control cells or of 0.1 mU/ml TSH treated cells (Figs 4a and b). The effect of 0.1 mM forskolin was unaffected when tested on control cells or was slightly decreased on TSH treated cells preincubated with TPA. Ten mU/ml TSH treated cells were desensitized against a further acute stimulation with 40 mU/ml TSH (Fig. 4c), but not against an acute stimulation with forskolin. The TPA pretreatment (2 hr) did not abolish the homologous desensitization to TSH and did not interfere with the acute forskolin effect on 10 mU/ml TSH treated cells. On 3 day cultured cells (Fig. 5), preincubation with TPA for 30 min significantly

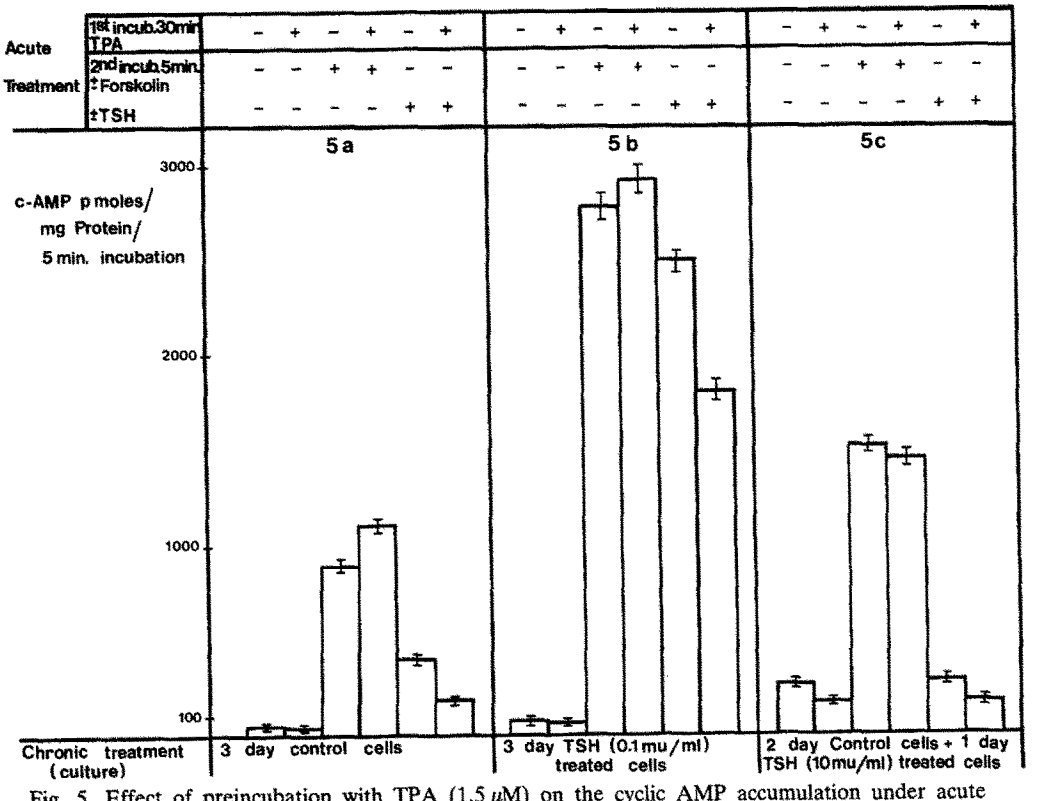

Fig. 5. Effect of preincubation with TPA (1.5 μM) on the cyclic AMP accumulation under acute subsequent incubation with TSH (40 mU/ml) or forskolin (0.1 mM) of 3-day cultured pig thyroid cells. Same conditions as in Fig. 4. (a, b) The statistical significance is as in 4a and 4b. (c) Not statistically significant: TPA vs control, TPA + forskolin vs forskolin, TPA + TSH vs TSH, TSH vs corresponding control. $P < 0.001$: forskolin vs control, TPA + forskolin vs TPA.

Table 1. Concentration responses of various phorbol derivatives on the inhibition of the cyclic AMP accumulation promoted by TSH and on the stimulation of the ^{32}P incorporation into the phosphatidylcholine (results of two experiments)

Phorbol derivative	Concentration	Inhibition of the TSH effect on cAMP accumulation (%)		Stimulation of the ^{32}P incorporation into phosphatidylcholine (control = 100%)	
TPA	1.5 nM	0	0	0	0
	15 nM	5	12	200	150
	150 nM	43	58	1050	850
	1.5 μM	62	80	1700	1550
Phorbol	from 1.5nM to 1.5μM	0	0	0	0
Phorbol 13-acetate	from 1.5nM to 1.5μM	0	0	0	0

Pig thyroid cells were cultured for 4 days with 0.1 mU/ml TSH. After washings, cells were incubated 2 hr in the absence or the presence of different concentrations of various phorbol derivatives. Then the incubation was pursued for 5 min with or without TSH (40 mU/ml) and IBMX (mM). Cyclic AMP was assayed as in Materials and Methods. The results were expressed as the percentage of inhibition of the TSH effect (2425 ± 180 pmoles/mg protein). ^{32}P incorporation into PC was determined after 2 hr incubation of the cells in the absence or in the presence of different concentrations of the various phorbol derivatives. Phospholipid analysis was effected as in Materials and Methods. The results were expressed as the percentage of stimulation of ^{32}P incorporation into PC (control = 2630 cpm/mg protein).

decreased the acute effects of 40 mU/ml TSH on control or on 0.1 mU/ml TSH treated cells (Figs. 5a and b). The acute effect of forskolin was not modified in control or in TSH treated cells preincubated with TPA.

The positive regulation of adenylate system was observed in 3-day, 0.1 mU/ml TSH treated cells stimulated acutely (5 min) either with TSH (40 mU/ml) or with forskolin (0.1 mM) (Fig. 5b). On the other hand, 2-day control cells desensitized by a 1-day treatment with 10 mU/ml TSH against an additional acute stimulation with TSH, were not desensitized against an acute forskolin stimulation (Fig. 5c). The TPA preincubation did neither modify the cells response nor abolish the desensitization to TSH.

In the same concentrations range as TPA, neither phorbol nor phorbol-13-acetate affected the thyroid metabolism (Table 1).

DISCUSSION

The treatment of cultured pig thyroid cells with the tumor promoting phorbol ester, TPA, deeply affects their morphology and their metabolism. The phospholipid labelling with ^{32}P-phosphate, which is an index of the turnover rate of the various species, is dramatically modified, the PC labelling being increased by 9 to 13 times. The TPA effect is combined to the general increase in the labelling due to the chronic effect of TSH during the culture period. The rise of the labelling ratio PI/PC elicited by TSH is almost completely masked by the higher labelling of PC promoted by TPA. The acute effect of TSH (40 mU/ml) disappears after 1–4 day culture in the presence of 0.1 mU/ml TSH [16]. When it is observed, i.e. on control cells, the acute effect of TSH is suppressed by the presence of TPA. Conversely, TSH acutely decreases the PC labelling promoted by TPA only in the situations where TSH is able to stimulate the PI labelling, i.e. mainly on 1 day control cells.

Other biochemical events linked to the activity of the thyroid cells are affected by the TPA treatment. Thus the iodinating capacity of the cells is enhanced by 100-fold after a 4-day chronic treatment with 0.1 mU/ml TSH; this increase is reduced tenfold by the presence of TPA during 45 min.

The cyclic AMP accumulation, promoted by a 5 min TSH (40 mU/ml) stimulation, is transiently increased by a short treatment with TPA (15 min) and thereafter inhibited by 50–60% by a 30 min or longer treatment. The inhibitory effect is observed in all physiological conditions of the cells: control-cells, cells sensitized of desensitized to TSH [21, 22]. Forskolin has been reported to act beyond the hormone receptor either directly on the catalytic or via the regulatory subunit of the adenylate cyclase [23–26]. The acute response of the cells to it is not affected significantly by a TPA treatment lasting between 30 min and 2 hr.

These results can be compared to those obtained by treating thyroid slices with Clostridium phospholipase C [27]. The phospholipid turnover was profoundly modified under those conditions: PC labelling was increased in response to its degradation

assessed by an enhanced amount of diacylglycerol. A similar result was obtained in embryonic muscle cells [28]. The specific phospholipid effect of TSH was removed by the phospholipase C treatment but could be partially restored, after a 3-hr incubation allowing the recovery of the PC content of the membrane. A decreased adenylate cyclase response to TSH [29] resulting in the decreased glucose oxidation [30] were also observed. These results were attributed to uncoupling of receptors, their binding capacity being not decreased [27].

A current hypothesis is that the TPA receptor and protein kinase C are related or identical molecules [31–34]. TPA binding to the diacylglycerol site of the protein kinase C can anchor the enzyme to the plasma membrane and increase its activity which in turn activates phospholipase A2 and the release of arachidonate from PC. This last possibility is supported by data of the literature [35–37] and is compatible with the effects of arachidonic [38] and eicosatetraynoic acid [39] in our system. Indeed these acids decrease altogether the cyclic AMP accumulation, the PI/PC labelling ratio of phospholipids and the iodinating capacity of 4-day TSH treated cells.

There are numerous recent reports on the effects of TPA in mammalian tissues. They are all interpreted as the result of phosporylations by protein kinase C. According to the nature of the target proteins and of the cell types, very disparate responses are produced. The phosphorylation of growth factor receptors (EGF, insulin, etc.) or TPA specific residues of serine and threonine counteracts EGF or insulin receptor autophosphorylation and EGF or insulin action [40, 41].

According to the studied systems, adenylate cyclase is inhibited [42, 43] or stimulated [44, 45] by a TPA treatment. In two cases the data support a phosphorylation of the guanine nucleotide binding protein, enhancing the cyclase activity in S49 lymphoma cells [46] and decreasing it in hepatocytes. [47].

Other enzymic activities implicated in PC metabolism are stimulated by a TPA treatment: a PC specific phospholipase C in human leukemic cells [48] and in Hela cells [49], the phosphatidate phosphohydrolase in adipocytes [50], the CTP: choline phosphate cytidylic transferase in myoblasts [51, 52]. This latter activity being regulated by phosphorylation-dephosphorylation [53].

The current knowledge about the TSH receptor mechanism [54] does not allow to study its eventual phosphorylation following a TPA treatment, but the actual effects on PC metabolism and adenylate cyclase activity are under investigation.

Note added in proof: A recent report from L. K. Bachrach, M. C. Eggo, W. W. Mak and G. N. Burrow, *Endocrinology* **116**, 1603–1609 (1985), shows that TPA (0.16 μM) stimulated cell growth, inhibited iodide uptake and organification and cyclic AMP accumulation.

Acknowledgements—We are grateful to Mrs Legué for the expert technical collaboration and to Mrs Subtil-François for typing the manuscript. We warmly thank the NIH (Bethesda) who provided us with thyrotropin and Sobevir (Rethel) who provided us with thyroid glands. This work was supported by grants from CNRS (ERA No 401) and INSERM (CRE 844008).

REFERENCES

1. P. M. Blumberg, *C.R.C. Critical Reviews-Toxicol.* **8**, 153 (1980).
2. M. Wigler and I. B. Weinstein, *Nature*, **259**, 232 (1976)
3. S. D. Schimmel and T. Hallam, *Biochem. biophys. Res. Commun.* **92**, 624 (1980).
4. G. R. Grotendorst and S. D. Schimmel, *Biochem. biophys. Res. Commun.* **93**, 301 (1980).
5. H. B. Padden and D. E. Vance, *Biochim. biophys. Acta* **620**, 636 (1980).
6. M. C. Cabot, C. J. Welsh, M. D. Callahan and E. Huberman, *Cancer Res.* **40**, 3674 (1980).
7. L. Levine and A. Hassid, *Biochem. biophys. Res. Commun.* **79**, 477 (1977).
8. R. I. Grove and S. D. Schimmel, *Biochim. biophys. Acta* **711**, 272 (1982).
9. Y. Takai, R. Minakuchi, U. Kikkawa, K. Sano, K. Kaibuchi, B. Yu, T. Matsubara and Y. Nishizuka, *Progr. Brain Res.* **56**, 287 (1982).
10. A. Sahai, K. B. Smith, M. Pannerselvam and D. S. Salomon, **109**, 1206 (1982).
11. A. S. Kraft, W. B. Anderson, H. L. Cooper and J. J. Sando, *J. biol. Chem.* **257**, 13193 (1982).
12. R. Stocker and C. Richter, *FEBS Letts.* **147**, 243 (1982).
13. D. J. Crutchley and J. R. Maynard, *Biochim. biophys. Acta* **762**, 76 (1983).
14. U. Lichti and M. M. Gottesman, *J. Cell Physiol.* **113**, 433 (1982).
15. J. Mauchamp, A. Margotat, M. Chambard, B. Charrier, L. Remy & M. Michel-Bechet, *Cell Tissue Res.* **204**, 417 (1979).
16. C. Gerard, B. Haye, C. Jacquemin and J. Mauchamp, *Biochim. biophys. Acta* **710**, 359 (1982).
17. H. L. Caila, M. S. Racine-Weisbuch and M. A. Delaage, *Analyt. Biochem.* **56**, 394 (1973).
18. B. Haye, G. Marcy and C. Jacquemin, *Biochimie* **61**, 905 (1979).
19. G. V. Marinetti and E. Stotz, *Biochim. biophys. Acta* **21**, 168 (1956).
20. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
21. N. Takasu, B. Charrier, J. Mauchamp and S. Lissitzky, *Eur. J. Biochem.* **90**, 139 (1978).
22. N. Takasu, B. Charrier, J. Mauchamp and S. Lissitsky, *Eur. J. Biochem.* **90**, 131 (1978).
23. D. Stengel, L. Guenet, M. Desmier, P. Insel and J. Hanoune, *Molec. Cell. Endocr.* **28**, 681 (1982).
24. J. E. Fradkin, H. Cook, M. C. Kilhoffer and J. Wolff, *Endocrinology* **111**, 848 (1982).
25. J. Van Sande, P. Cochaux and J. E. Dumont, *FEBS Letts.* **150**, 137 (1982).
26. J. Van Sande, P. Cochaux, J. Mockel and J. E. Dumont, *Molec. Cell Endocr.* **29**, 109 (1982).
27. B. Haye and C. Jacquemin, *FEBS Letts.* **18**, 47 (1971).
28. C. Kent, *Proc. natn. Acad. Sci. U.S.A.* **76**, 4474 (1979).
29. K. Yamashita, G. Bloom, B. Rainard, U. Zor and J. B. Field, *Metabolism* **19**, 1109 (1970).
30. J. E. Dumont and R. Burelle, *C. R. Soc. Biol.* **158**, 2500 (1964).
31. J. J. Sando and M. C. Young, *Proc. natn. Acad. Sci. U.S.A.* **80**, 2642 (1983).
32. K. L. Leach, M. L. James and P. M. Blumberg, *Proc. natn. Acad. Sci. U.S.A.* **80**, 4208 (1983).
33. J. E. Niedel, L. J. Kuhn and G. R. Vanderbark, *Proc. natn. Acad. Sci. U.S.A.* **80**, 36 (1983).
34. M. Castagna, Y. Takai, K. Katbuchi, J. Sand, U. Kikkawa and Y. Nishizuka, *J. biol. Chem.* **257**, 7847 (1982).

35. D. L. Lasking, J. D. Laskin, I. B. Weinstein and R. A. Carchman, *Cancer Res.* **40**, 1028 (1980).
36. M. Ganss, D. Seemann, G. Forstenberger and F. Marks, *FEBS Letts.* **142**, 54 (1982).
37. J. L. Humes, S. Sadowski, M. Galavage, M. Goldenberg, E. Subers, R. J. Bonney and F. A. Kuehl Jr., *J. biol. Chem.* **257**, 1591 (1982).
38. C. Gerard, B. Haye and C. Jacquemin, *FEBS Let* **132**, 23 (1981).
39. B. Haye, C. Gerard and C. Jacquemin, *FEBS Letts.* **148**, 281 (1982).
40. C. Cochet, G. N. Gill, J. Meisenhelder, J. A. Cooper and T. Hunter, *J. biol. Chem.* **259**, 2553 (1984).
41. S. Takayama, M. F. White, V. Lauris and C. R. Kahn, *Proc. natn. Acad. Sci. U.S.A.* **81**, 7797 (1984).
42. C. Rochette-Egly and M. Castagna, *FEBS Letts.* **103**, 38 (1979).
43. M. A. Brostrom, C. D. Brostrom, L. A. Brotman, C. Lee, D. J. Wolff and H. M. Geller, *J. biol. Chem.* **257**, 6758 (1982).
44. R. Simantov and L. Sachs, *Biochim. biophys. Acta* **720**, 120 (1982).
45. J. P. Perchellet and R. K. Boutwell, *Cancer Res.* **40**, 2653 (1980).
46. J. D. Bell, I. L. O. Buxton and L. L. Brunton, *J. biol. Chem.* **260**, 2625 (1985).
47. C. M. Heyworth, A. D. Whetton, A. R. Kinsella and M. D. Houslay, *FEBS Letts.* **170**, 38 (1984).
. M. W. Lockney, H. M. Golomb and G. D. Dawson, *Biochim. biophys. Acta* **796**, 384 (1984).
49. G. R. Guy and A. W. Murray, *Cancer Res.* **42**, 1980 (1982).
50. M. Hall, S. J. Taylor and E. D. Saggerson, *FEBS Letts.* **179**, 351 (1984).
51. S. A. Hill, W. C. McMurray and B. D. Sanwal, *Can. J. Biochem. Cell Biol.* **62**, 369 (1984).
52. D. E. Vance and S. L. Pelech, *Trends Biochem. Sci.* **9**, 17 (1984).
53. S. L. Pelech and D. E. Vance, *J. biol. Chem.* **257**, 14198 (1982).
54. T. B. Nielsen, Y. Totsuka, E. S. Kempner and J. D. Field, *Biochemistry* **23**, 6009 (1984).