Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 323-329

ROLE OF CALCIUM ON TPA-INDUCED SECRETION OF ACTH AND PGE_2 BY PITUITARY CELLS: EFFECT OF DEXAMETHASONE

Evelyne Dartois and Marie M. Bouton

Centre de Recherche Roussel UCLAF
111 route de Noisy.
93230 Romainville, France

Received May 22, 1986

SUMMARY: In pituitary cells in primary culture, ACTH and PGE_2 secretions can be simultaneously stimulated by TPA, in the presence of Ca^{2+}. However, whilst PGE_2 secretion is under an absolute requirement for Ca^{2+}, ACTH secretion is not. Both secretions are inhibited by dexamethasone but to various extents: PGE_2 release is abolished in the presence of dexamethasone whilst only 35% of the TPA -induced ACTH release is sensitive to dexamethasone. Similar inhibitory effects are observed with mepacrine, a PLA_2-inhibitor, suggesting that PLA_2-activation could be related to these secretory processes. Since PLA_2-inhibition by dexamethasone is claimed to be mediated via lipocortin, these results suggest that a lipocortin-like protein is present in pituitary cells and could be involved in the TPA-induced secretions of PGE_2 and ACTH.

Phorbol esters, such as 12-O-tetradecanoylphorbol 13-acetate (TPA), activate numerous cellular responses including cell growth and hormone secretion (1,2) by activating the calcium and phospholipid-dependent enzyme protein kinase C (3,4). For instance, in normal and neoplastic pituitary cells, TPA induces ACTH secretion, to an extent similar to that triggered by factors such as CRF and forskolin whose effects are mediated via the cAMP-dependent protein kinase system (5,6). In fibroblasts, TPA is able to induce the release of arachidonic acid from membrane phospholipids and its conversion into prostaglandins (7). These TPA-induced secretions (ACTH in pituitary cells and prostaglandins in fibroblasts) are both inhibited by dexamethasone, a potent glucocorticoid agonist (6,8) that is thought to inhibit prostaglandins secretion, by increasing the synthesis of a

Abbreviations: TPA, 12-O-tetradecanoylphorbol 13-acetate; ACTH, adrenocorticotropin; PGE_2, prostaglandin E_2; PLA_2, phospholipase A_2; CRF, corticotropin releasing factor; BSA, bovine serum albumin.

0006-291X/86 $1.50

protein (lipocortin) inhibitor of the enzyme phospholipase A_2 (PLA_2) responsible of the liberation of arachidonic acid from membrane phospholipids (9-11).

In order to establish whether the inhibitory effects of dexamethasone on ACTH and PGE_2 release are mediated via the same intracellular processes, we have compared the stimulatory effect of TPA on both ACTH and PGE_2 secretions and the inhibitory effect of dexamethasone on these induced secretions in rat pituitary cells in primary culture. Since the effect of TPA involves a Ca^{2+}-dependent kinase and that Ca^{2+} has been shown to activate PLA_2 (12), we have also examined the role of Ca^{2+} on these secretions.

MATERIALS AND METHODS

Materials: Medium 199 (with 1.8 mM Ca^{2+} and without Ca^{2+}) and fetal calf serum were obtained from Flow Laboratories, Hank's salt solution and penicillin/streptomycin from Gibco, trypsin and TPA from Sigma, forskolin from Calbiochem Hoechst, ionophore A23187 from Boehringer Mannheim and mepacrine from Boots. Dexamethasone was synthetised at the Roussel-Uclaf Research Center.

Preparation of solutions: Ionophore A23187 was initially dissolved in dimethylsulfoxide to achieve a concentration of 0.1 M. Forskolin, dexamethasone, TPA were dissolved in ethanol. Subsequent dilutions were prepared in medium 199. Control cultures received ethanol and/or dimethylsulfoxide at the same dilution. These concentrations of ethanol or dimethylsulfoxide had no effect on basal or induced secretion of ACTH and PGE_2. The complete culture medium consisted in medium 199 supplemented with 0.3% of bovine serum albumin (BSA) and 0.5% of a penicillin/streptomycin solution (solution: 10 000 units/ml of penicillin and 10 mg/ml of streptomycin).

Preparation of Dispersed Anterior Pituitary Cells: Pituitary dispersion was performed according to Vale et al. (13) with slight modifications : adult male Sprague-Dawley rats (190-210g) were decapitated and their pituitary glands were immediately removed. Pituitaries (10 glands) were rinsed twice with 5 ml of Hank's salt solution containing 0.3% BSA maintained at room temperature. The neurohypophyses were removed and the adenohypophyses were finely minced in a Petri dish containing 5 ml of complete medium, then transferred to a 50 ml centrifugation tube and allowed to settle. The medium was decanted and replaced twice to remove the remnants of lysed cells disrupted during mincing of the tissue. The adenohypophyses were then disrupted at 37°C with vigorous shaking for three successive 15 min periods in 10 ml of complete medium containing 0.25% trypsin. Every 15 min the fragments were drawn in and out of a Pasteur Pipette. Finally the solution was filtered through organza cloth to remove residual tissue fragments. Complete medium (10 ml) supplemented with 10% fetal calf serum was added to the filtrate to inhibit trypsin. After centrifugation at 800g for 10 min, the cell pellet thus obtained was resuspended in complete medium supplemented with 10% of fetal calf serum. The cells were counted with the aid of a hemocytometer and their viability was tested by trypan blue exclusion.

Effects of Test-Compounds on ACTH and PGE_2 Secretion: 5.10^5 dispersed cells in complete medium supplemented with 10% of fetal calf serum were distributed in Linbro multiwell Petri dishes and maintained for two days at 37°C under 5% CO_2-95% air. After this period, the medium was removed and replaced by complete medium without BSA but supplemented with 10% fetal calf serum. On day 5 of the culture, test-compounds (forskolin, A23187 and TPA) were added in 1 ml of medium 199. Four hours later, media were removed by aspiration. One part was stored frozen until PGE_2 determination, the other part was acidified with 3% HCl N before freezing and kept frozen until ACTH determination.

Test-compound activity was expressed as the compound concentration giving a half-maximal response (ED_{50}).

Effects of Dexamethasone on Induced ACTH and PGE_2 Secretion: Various concentrations(0.1nM-1μM) of dexamethasone were added to the medium 1 hour before the addition of test-compounds. Dexamethasone activity was expressed as the dexamethasone concentration producing a half-maximal inhibition of ACTH and PGE_2 secretion (IC_{50}).

ACTH and PGE_2 Determination: The concentration of ACTH in the culture medium was measured by radioimmunoassay using an antibody supplied by Bioproduct. The sensitivity of this double antibody assay was 20 pg per tube. PGE_2 concentration was determined by a radioimmunoassay, as previously described (14) using rabbit anti-PGE_2 antiserum (Pasteur Institute, Paris, France) and a method involving overnight incubation at 5°C followed by a separation of the bound and free compound by dextran-coated charcoal. The sensivity of the assay was 1 pg per tube.

RESULTS

Effect of A23187 on ACTH and PGE_2 release: At none of the concentrations tested (1 nM-10 μM) did the ionophore A23187 modify ACTH secretion; this is in contrast to previously published results with tumor pituitary cells (15,16). On the contrary, addition of A23187 to the culture medium led to marked PGE_2 release into the medium. In the presence of 1.8 mM Ca^{2+}, the PGE_2 release depended upon the concentration of added A23187 (ED_{50} = 0.3 μM). A 10 μM concentration of A23187 led to a maximal 12-fold increase in PGE_2 (Fig.1.A). This effect was Ca^{2+} dependent since in a Ca^{2+}-free medium, A23187 was inactive (Tab.1).

Dexamethasone, added 1h before A23187, inhibited PGE_2 release only if A23187 concentrations were lower than 0.5 μM. With 0.4 μM A23187, the inhibitory effect of dexamethasone was dose dependent

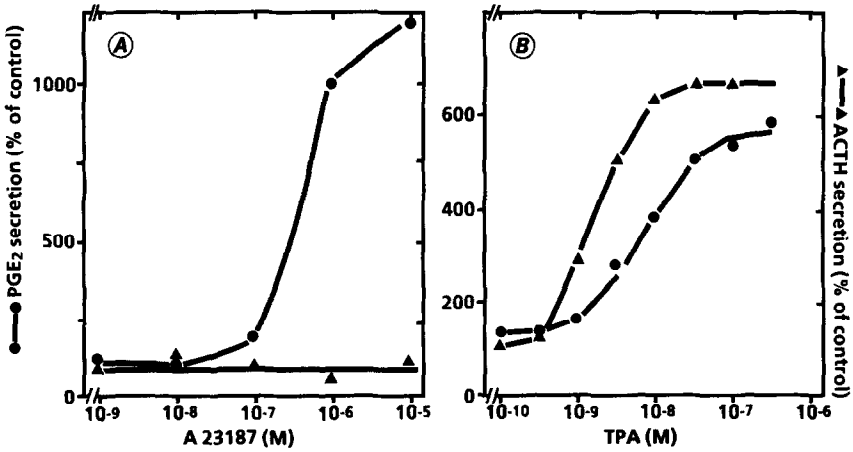

Fig.1. Effect of A23187 and TPA on ACTH and PGE_2 secretion. Pituitary cells in primary culture (5 10^5 cells/dish) were incubated for 4h in the presence of the indicated concentrations of A23187 (A) or TPA (B). control value = 5ng/ml for ACTH (▲) and 0.3ng/ml for PGE_2 (●).

Table 1

	PGE_2 release (ng/ml)		ACTH release (ng/ml)	
	-DEX	+ DEX	-DEX	+ DEX
Control	0.42 ± 0.03	ND	0.77 ± 0.07	ND
Control + Ca^{2+}	0.6 ±0.1	0.38 ± 0.03	2.7 ± 0.2	1.5 ± 0.03
TPA.	0.69 ± 0.08	0.45 ± 0.08	10.2 ± 0.9	6.7 ± 0.6
TPA + Ca^{2+}	2.4 ± 0.1	0.79 ± 0.01	21. ± 1.	16. ± 2.
A 23187.	0.52 ± 0.01	0.53 ± 0.09	0.6 ± 0.06	0.6 ± 0.1
A23187 + Ca^{2+}	4.2 ± 0.4	ND	0.83 ± 0.2	ND
A23187 + TPA.	1.9 + 0.1	1.28 ± 0.05	7.4 ± 0.5	7.35 ± 0.02
A23187 + TPA + Ca^{2+}.	12. ± 1.6	6.8 ± 0.8	5.0 ± 0.4	9.0 ± 1.

Effect of Ca^{2+} on ACTH and PGE_2 release. Pituitary cells were preincubated for 1h with or without 50 nM dexamethasone, in medium199 without Ca^{2+} or with 1.8 mM Ca^{2+}. Then cells were incubated for 4h with 0.1 µM TPA and/or with 0.4 µM A23187. The results were given as mean ± S.E.M. of three different wells.

(IC_{50} = 1.6 nM),(Fig.2.A). A 100 nM concentration of dexamethasone led to a maximal 65% inhibition of PGE_2 release. A similar inhibition was obtained with 50 µM mepacrine, a well-known inhibitor of PLA_2 activity (17), suggesting that this PGE_2 secretion was related to an activation of the Ca^{2+}-dependent enzyme PLA_2.

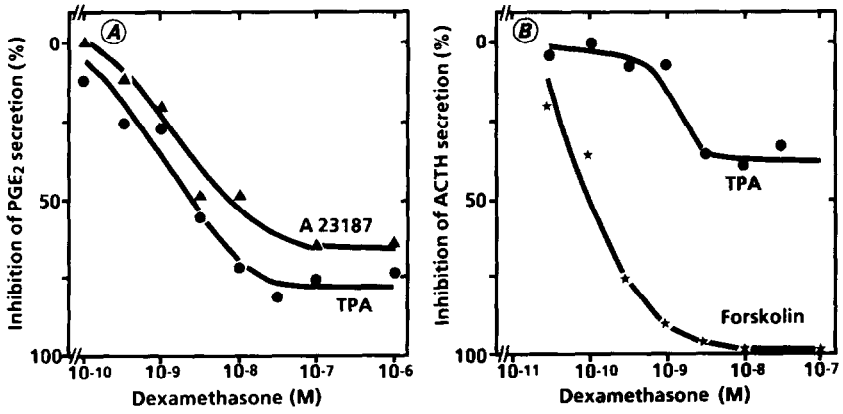

Fig.2. Effect of dexamethasone on ACTH and PGE_2 secretion. Cells were preincubated for 1h with the indicated concentrations of dexamethasone, and then incubated for 4h in the presence of 0.4 µM A23187 (▲) or 0.1 µM TPA(●) or 10 µM forskolin (★). Results are expressed as percent inhibition of the stimulation. (A) PGE_2 basal release was 0.3ng/ml, TPA increased it to 1.6ng/ml, and PGE_2 basal release was 0.7ng/ml, ionophore increased it to 3.5ng/ml. (B) ACTH basal release was 5ng/ml, TPA increased it to 33ng/ml and ACTH basal release was 7ng/ml, and forskolin increased it to 36ng/ml.

Effect of TPA on ACTH and PGE_2 release: TPA stimulated in a dose-related manner the secretion of both ACTH and PGE_2 (Fig.1.B). In the presence of 1.8 mM Ca^{2+} in the medium, a 100 nM TPA concentration was necessary to achieve maximal PGE_2 release. This effect was quantitatively similar to that induced by 0.4 µM A23187. The TPA-induced release of PGE_2 was under an absolute requirement for Ca^{2+}: in a Ca^{2+}-free medium TPA alone did not significantly increase PGE_2 secretion (Tab.1). However, TPA-induced stimulation of PGE_2 was restored if A23187 (0.4 µM) was added to the incubation medium.

Pretreatment of the cells for 1h with dexamethasone led to a maximal 80% inhibition of TPA-induced PGE_2 release (Fig.2.A). This inhibition was dose-dependent (IC_{50}= 1.6 nM, a value comparable to that obtained on A23187-induced PGE_2 release). A similar inhibition was achieved when cells were preincubated with 50µM of the PLA_2 inhibitor mepacrine. When PGE_2 release was induced by both TPA and ionophore, whether in the presence or in the absence of extracellular Ca^{2+}, dexamethasone was slightly less potent since an inhibition of only 44% was observed with a 50 nM concentration of dexamethasone (Tab.1).

In the presence of 1.8 mM Ca^{2+} maximal release of ACTH was observed for a TPA concentration of 10 nM (Fig.1.B). This stimulation was quantitatively higher than that triggered by 10 µM forskolin (7-fold increase vs 5-fold with forskolin, results not shown). This ACTH secretion did not seem to be dependent on extracellular Ca^{2+}. In a Ca^{2+} free medium the percentage of stimulation by TPA was similar to that observed in the presence of 1.8 mM Ca^{2+} (Tab.1), in spite of the lower basal ACTH levels in Ca^{2+} free medium, the concentration of ACTH released was lower. In contrast to PGE_2 release, ACTH release was not increased by the adddition of A23187 to the Ca^{2+}-free medium. In certain experiments, a slight inhibitory effect was observed.

In the presence of 1.8 mM Ca^{2+}, addition of dexamethasone to the culture medium led to an inhibition of TPA-induced ACTH secretion. This inhibition was dose-dependent (IC_{50} = 1.6 nM, a value similar to that observed on forskolin-induced secretion) (Fig.2.B). But, while dexamethasone was able to inhibit completely forskolin-induced ACTH release, it had only a partial inhibitory

effect (35%) on TPA-induced ACTH release as observed with mepacrine which had no effect on the forskolin-induced ACTH secretion.

DISCUSSION

Our results show that, in rat pituitary cells in primary culture, TPA can stimulate both ACTH and PGE_2 secretion, however, the pathways of stimulation seem to be different since, while PGE_2 secretion was under an absolute requirement for Ca^{2+}, ACTH secretion was not. In a Ca^{2+}-free medium, TPA can induce PGE_2 secretion only if A23187 was added, while, under the same conditions, ACTH secretion was not modified. These results suggest that in pituitary cells, like in other systems (18,19,20), mobilization of intracellular Ca^{2+}, while ineffective by itself (no effect of A23187 alone in a Ca^{2+}-free medium), is a prerequisite for the induction of PGE_2 release by TPA. It does not seem necessary for the induction of ACTH responses. Both ACTH and PGE_2 secretions induced by TPA, can be inhibited to various extents by dexamethasone: PGE_2 release can be totally inhibited while ACTH release is inhibited up to 35% only. In both cases, mepacrine, a PLA_2-inhibitor has the same effect as dexamethasone. Thus, TPA-induced PGE_2 release and 35% of ACTH release could be related to an activation of PLA_2.

It has been shown that, the inhibitory effect of dexamethasone on PLA_2 is due to its ability to provoke the synthesis of a 40 kD protein, lipocortin (21), which forms a complex with PLA_2 rendering the latter inactive (10). Activation of PLA_2 can be achieved by dissociation of the complex PLA_2-lipocortin by phosphorylation of lipocortin. TPA is thought to act by binding to and activating protein kinase C leading to the phosphorylation of specific protein presumed to be necessary for eliciting the full response and Hirata has shown that in thymocytes lipocortin phosphorylation can be induced by kinase C activation (22). Thus, the PGE_2 secretion and at least part of the ACTH secretion, induced by TPA could be due to the phosphorylation of a lipocortin-like protein thus allowing PLA_2 to liberate arachidonic acid from membrane phospholipids. Experiments are in progress to verify that a lipocortin-like protein is effectively present in pituitary cells and could play a role in the secretory processes at the pituitary level.

Acknowledgements: We are grateful to Dr F. Russo Marie and Dr M. Gervais for their helpful advice.

REFERENCES

1. Nishizuka, Y. (1984) Nature. 308, 693-698.
2. Berridge, M. J., and Irvine, R. F. (1984) Nature. 312, 315-321.
3. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U., and Nishizuka, Y. (1982) J.Biol.Chem. 257, 7847-7851.
4. Uratsuji, Y., Nakanishi, H., Takeyama, Y., Kishimoto, A., and Nishizuka, Y. (1985) Biochem. Biophys. Res. Commun. 130, 654-661.
5. Vale, W., and Rivier, C. (1977) Federation Proceedings 36, 2094-2099.
6. Abou-Samra, A., Catt, K. J., and Aguilera, G. (1986) Endocrinology. 118, 212-217.
7. Mufson, R. A., DeFeo, D., and Weinstein, I.B. (1979) Mol. Pharm. 16, 569-578.
8. Chandrabose, K. A., Lapetina, E. G., Schmitges, C.J., Siegel, M. I., and Cuatrecasas, P. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 214-217.
9. Blackwell, G. J., Carnuccio, R., Di Rosa, M., Flower, R. J., Ivanyi, J., Langham, C. S. J., Parente, L., Persico, P., and Wood, J. (1983) Adv. in Prost. Thromb. and Leuk. Research. 11, 65-71.
10. Hirata, F. (1983) Adv. in Prost. Thromb. Leuk. Research. 11, 73 -78.
11. Rothhut, B., Cloix, J. F., and Russo-Marie, F. (1983) Adv. in Prost. Thromb. and Leuko. research. 1983, 51-56.
12. Billah, M. M., Lapetina, E. G., and Cuatrecasas, P. (1980) J. Biol. Chem. 255, 10227-10231.
13. Vale, W., Grant, G., Amoss, M., Blackwell, R., and Guillemin, R. (1972) Endocrinology. 91, 562-572.
14. Dray, F., Charbonnel, B., and Maclouf, J. (1975) Europ. J. Clin. Invest. 5, 311-318.
15. Richardson, U. I. (1983) Endocrinology. 113, 62-68.
16. Miyazaki, K., Reisine, T., and Kebabian, J. W. (1984) Endocrinology. 115, 1933-1945.
17. Vigo, C., Lewis, G. P., and Piper, P. J. (1979) Bioch. Pharm. 29, 623-627.
18. Mobley, A., and Tai, H. (1985) Biochem. Biophys. Res. Commun. 130, 717-723.
19. Sakamoto, C., Matozaki, T., Nagao, M., and Baba, S. (1985) Biochem. Biophys. Res. Commun. 131, 314-319.
20. Ruskoaho, H., Toth, M., and Lang, R. E. (1985) Biochem. Biophys. Res. Commun. 133, 581-588.
21. Wallner, B. P., Mattaliano, R. J., Hession, C., Cate, R. L., Tizard, R., Sinclair, L. K., Foeller, C., Chow, E. P., Browning, J. l., Ramachandran, K. L., and Pepinsky, R. B. (1986) Nature. 320, 77-81.
22. Hirata, F., Matsuda, K., Notsu, Y., Hattory, T., and Del Carmine, R. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 4717-4721.