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OSCILLATING LEVELS OF ADENYLATE AND GUANYLATE CYCLASE ACTIVITIES IN RAT EMBRYO FIBROBLASTS STIMULATED TO DIVIDE

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Summary : Basal activities of membrane-bound adenylate and guanylate cyclase were determined in confluent rat embryo cells stimulated to proliferate by either the renewal of serum-supplemented growth medium or the addition of a mitogen, the 12-O-tetradecanoyl-phorbol-13-acetate (TPA). A transient increase in guanylate cyclase activity was observed within minutes following either treatment while adenylate cyclase activity either abruptly declined in serum-stimulated cells or remained unaffected in TPA-treated cells. In response to both mitogenic treatment, adenylate and guanylate cyclase activities varied reciprocally throughout the pre-replicative phase up to DNA synthesis. The lower levels of guanylate over adenylate activity ratio occurred prior to the onset of the replicative phase whereas the higher levels were coincident with DNA synthesis. A similar pattern of oscillating levels of sodium-fluoride-stimulated adenylate and lubrol-treated guanylate cyclase activities was observed.

Experimental manipulation as well as growth-state-related changes of basal levels in cultured cells have led to implicate cyclic AMP[1] and cyclic GMP in the control of cell division as reviewed in Ref. 1,2. Previous studies suggested that the role of cyclic nucleotides in the regulation of cell proliferation seems to depend on the occurrence of a critical level of those nucleotides at specific stages of the cell cycle (3,4). Recently, large and reciprocal fluctuations in the intracellular levels of cyclic nucleotide occurring during passage through the mitotic cycle have been reported (5). To date, little information is available about the underlying mechanism responsible for such alterations in cyclic nucleotide levels. It may involve the enzymes governing the metabolism of cyclic nucleotides, adenylate and guanylate cyclases or cyclic nucleotide phosphodiestase as well as the exclusion process which also controls the intracellular levels of those nucleotides (5,6).

1. Abbreviations used are : TPA,12-O-tetradecanoyl-phorbol-13-acetate; cyclic GMP, cyclic guanosine 3' :5'-monophosphate ; cyclic GMP,cyclic adenosine 3':5'-monophosphate ;PBS, phosphate buffered saline ;DTT,dithiothreitol.

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The present study was undertaken in order to determine the levels of adenylate and guanylate cyclase activities as cells entered into the mitotic cycle. The experiments were performed in confluent cultures of rat embryo fibroblasts released from quiescence by two mitogenic treatments. Density-inhibited cells were stimulated to grow either by the addition of serum associated with a medium change or by treatment with the tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate, of which the activity has been generally associated with hyperplasia (7). We described here the oscillations of adenylate and guanylate cyclase activities which occur during the pre-replicative and replicative phases following the stimulation of rat embryo fibroblasts.

MATERIALS AND METHODS

Chemicals : (^{3}H) cyclic GMP and (^{3}H) cyclic AMP were obtained from New England Nuclear Corporation (Boston, Mass, USA) and TPA was purchased from Consolidated Midland Co.

Cell cultivation and treatment : the experiments were carried out with secondary cultures of rat embryo cells obtained according to the previously described technique (8). Cells were seeded at a density of 2 x 10^5 cells per ml in 60 mm Petri dishes containing 5 ml of Dulbecco's growth medium H 16 (GIBCO, Paisley,Scotland), supplemented with 10 % fetal calf serum and incubated at 37° C in a humidified atmosphere. At day six, when the cells have become confluent, the used growth medium was replaced with serum-supplemented fresh medium containing or not freshly dissolved TPA at a final concentration of 0.1 ug/ml. In parallel experiments, TPA as solution in acetone was added directly to the dishes without changing the medium. At appropriate intervals after treatment the cells were rinsed three times with ice-cold PBS and scrapped from the dish with a rubber policeman in a small volume of PBS. The cells were broken by the freezing-thawing procedure in a buffer containing 250 mM sucrose, 1 mM DTT and 25 mM Tris-HCl at pH 7.5. The particulate fraction was collected as a pellet by a 20 min. centrifugation at 20,000 g and resuspended in the original buffer. Aliquots of the particulate suspension were assayed for adenylate and guanylate cyclase activities and for the protein content according to Lowry et al (9).

Adenylate and guanylate cyclase assays : Both cyclase activities were performed at 37° C for 10 min. and the incubation mixtures were essentially as described by Levilliers et al (10). The standard reaction mixture contained 25 mM Tris-HCl pH 7.5, 1 mM DTT, 10 mM creatine phosphate and 0.1 mg per ml of creatine kinase in a final volume of 0.1 ml. In addition, the assay mixture for guanylate cyclase contained 2.5 mM $MnCl_2$ plus 0.5 mM ATP. As indicated, adenylate cyclase activity was assayed in presence of 10 mM sodium fluoride and guanylate cyclase activity in presence of 0.25 % lubrol. Reaction was initiated by adding 20 to 60 µg of enzyme preparation in a 20 µl volume and was terminated by heating at 90 ° C in 0.4 ml of 50 mM sodium acetate buffer, pH 4.0 containing 20 mM EDTA. Formed cyclic GMP and cyclic AMP were assayed according a previously described protein binding assay(11).

Cyclic nucleotide production was found to be linear with protein concentration up to 1 mg per ml and with time up to 30 min. at 37°C. In order to evaluate the extent of cyclic nucleotide degradation during the course of the cyclase reaction, a given amount of (^{3}H) cyclic AMP or, (^{3}H) cyclic GMP was

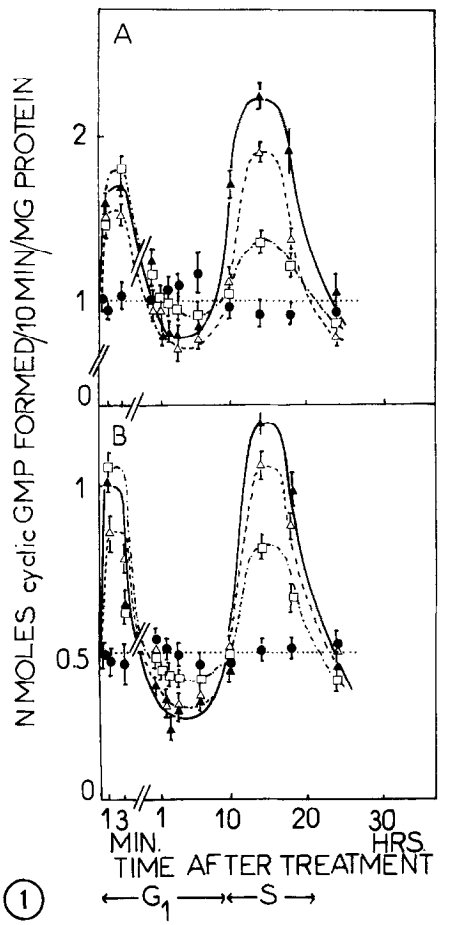
added to the reaction mixture and incubated under the assay conditions for cyclase activity. Recovered cyclic nucleotides were purified by thin-layer chromatography in ethanol-1M ammonium acetate, pH 7.4-0.1 M EDTA,pH 8.2 (70,30,1) and analyzed for radioactivity. It was concluded from these experiments that neither the formed cyclic AMP nor the formed cyclic GMP were significantly degraded under the assay conditions. In addition, care was taken that no component of the cyclase assays interfered with the cyclic nucleotide assay at the used concentrations.

RESULTS

Previous study has shown that density-inhibited rat embryo cells in secondary culture were released from quiescence by either the addition of serum associated with a growth medium change or treatment with 0.16 μM TPA (12). The incorporation of (^{3}H)-thymidine into DNA started by 9 hrs and reached maximal values around 15 hrs after the addition of either mitogenic agent.The magnitude of the response was approximately additive when both treatments were combined.

Membrane-bound adenylate and guanylate cyclase activities were assayed in cells committed to entry into the mitotic cycle and progressing through the pre-replicative (G_1) and replicative (S) phases. As depicted in Fig. 1B, basal guanylate cyclase activity doubled briefly within minutes following a growth medium change, fell below the inital level by 1 to 6 hrs and reached again high levels during the S-phase. Adenylate cyclase activity exhibited a distinct overall pattern of variations, as shown in fig. 2B : the activity decreased abruptly and remained at lower values through the pre-replicative and replicative phases, except for a marked increase which was observed between 1 to 6 hrs. Sodium fluoride at the concentration of 10 mM provoked a 5-fold stimulation of the adenylate cyclase activity whereas the addition of 0.25 % lubrol in the reaction mixture doubled the guanylate cyclase activity. As shown in Fig.1 and 2,basal and stimulated activities oscillated in a parallel manner.

Confluent cells stimulated to divide by the addition of TPA directly to the cultures without a medium change, exhibited a roughly similar pattern of enzyme activities although the magnitude of the oscillations was somewhat different. In contrast with the serum stimulation, which elicited a marked decline in ade-



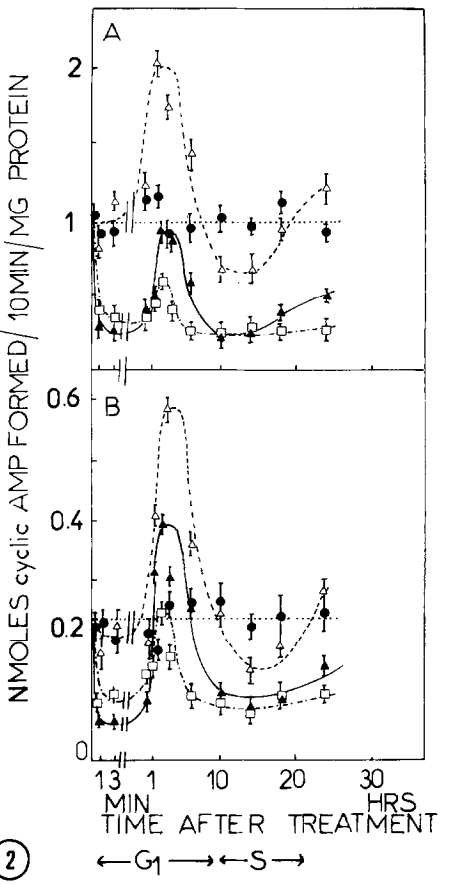


FIG. 1 : Membrane-bound guanylate cyclase activity of confluent rat embryo fibroblasts stimulated to proliferate by treatment with 0.16 µM TPA (Δ--Δ) or a change of the 10 % serum-supplemented growth medium containing (▲—▲) or not (□·-·□) 0.16 µM TPA. Controls were either untreated or treated with a volume of acetone identical to that added to the TPA-treated cultures (●···●). A, guanylate cyclase basal activity ; B,0.25 % lubrol-treated guanylate cyclase activity. Each value is the average of triplicate determinations on two different dishes.

FIG. 2 : Membrane-bound adenylate cyclase activity of confluent rat embryo fibroblasts stimulated to proliferate. See Fig. 1 for key to symbols. A, adenylate cyclase basal activity ; B, 10mM sodium fluoride-stimulated adenylate cyclase activity. Each value represents the average of triplicate determinations on two different dishes.

nylate cyclase activity, TPA did not affect the enzyme activity during the early pre-replicative phase and consequently the later variations of the enzyme activity occurred at higher levels than those of serum-stimulated cells The combined treatment with the two mitogenic agents generated a pattern of

oscillations of both cyclase activities similar to that induced by each agent alone. The magnitude of the oscillations reflected the maximal variations attained by either agent alone with the exception that an additive increase in guanylate cyclase activity was obtained in S-phase.

Acetone, the solvent of TPA, altered neither the adenylate or guanylate cyclase activities of unstimulated controls nor the serum-induced fluctuations of both enzyme activities during the 24 hrs duration of the experiments.

It should be stressed that the mid and late oscillations in the levels of both enzymes varied reciprocally regardless of the applied mitogenic treatment to reinitiate growth. In order to summarize the foregoing results the fluctuations of the basal activity ratio of guanylate cyclase over adenylate cyclase in rat embryo fibroblasts during the 24 hrs following serum or TPA stimulation have been depicted Fig. 3. It should be noted that the activity ratio of unstimulated cells at confluency remains at a steady level whereas three discrete periods could be distinguished when fibroblasts entered into the mitotic cycle. The early pre-replicative and the replicative phases are characterized by predominence of the guanylate cyclase activity whereas the adenylate cyclase activity was pre-eminent in mid to late pre-replicative phase.

DISCUSSION

Membrane-bound adenylate and guanylate cyclase activities, which approximately represent 80 % of the overall cyclase activities (unpublished data) were studied following the induction of density-inhibited fibroblasts to proliferate. The present report describes the occurrence of large and mainly reciprocal oscillations in the levels of both enzyme activities when cells entered into the mitotic cycle up to the onset of DNA synthesis.

We previously studied the levels of both cyclic nucleotides in rat embryo fibroblasts undergoing similar treatments. The variations in either cyclase activities resulting of cell release from quiescence paralleled the variations in levels of the respective cyclic nucleotide (13). Parallel experiments to those reported herein were conducted in exponentially growing cells which exhi-

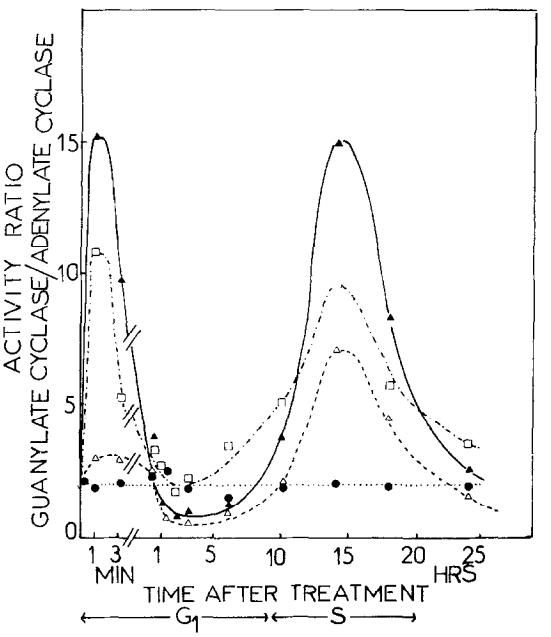


FIG. 3 : Ratio of guanylate cyclase over adenylate cyclase basal activity in confluent rat embryo fibroblasts either untreated (●···●) or stimulated to proliferate with 0.16 μM TPA (Δ-·-Δ) or a medium change associated (▲—▲) or not (□·–·□) with 0.16 μM TPA treatment.

bit two peaks of increased DNA synthesis in response to a growth medium change or after exposure to TPA, the first which occurred immediatly may involve cells at the G_1/S boundary whereas the later looked similar to that observed in cells released from quiescence (12). Again, the pattern of oscillations in cyclase activities correlated closely with the pattern of cyclic nucleotides levels (data not shown). The presented results as well as previously reported similar observations on adenylate cyclase (14) and very recently on guanylate cyclase activity (15) favor the hypothesis that the synthetic enzymes play a major role in the regulation of the intracellular levels of cyclic nucleotides . It should be pointed out that any conclusion regarding the correlation between the cyclic nucleotide content and the activity of cyclase preparations would assume that changes in cyclase activity of intact cells are related with in vitro changes in cyclase activities from broken preparations, and that the activity

ratio of both enzymes, as measured in vitro ,is related with the actual ratio. The control values indicated that the cyclic GMP synthesis was faster than that of cyclic AMP whereas the intracellular concentration of the latter was about 10-fold over that of the former nucleotide (13). A possible candidate for catalyzing a cyclic GMP degradation faster than that of cyclic AMP may be the activator-sensitive phosphodiesterase which has a high apparent affinity for cyclic GMP and a relatively low apparent affinity for cyclic AMP (16).

The results emphasize that the early fluctuations of adenylate cyclase activity are distinct in regard of the applied mitogen. The rapid decline which occurs in resting cells stimulated by a medium change was not observed in TPA-treated cells supporting the evidence that an early drop in cyclic AMP was not required for reinitiation of growth. Simultaneously, a transient rise of guanylate cyclase activity occurs following either mitogenic agent , that which favors the possibility, already suggested (17),that increased levels of cyclic GMP may be required for the initiation of growth.

The antagonist relationship between both cyclases generally observed following stimulation of rat embryo fibroblasts to divide supports the dualism concept of biological regulations, through the opposing actions of cyclic AMP and cyclic GMP, as proposed by Goldberg et al (18) and raises the question of the involved mechanism in such a coordinate response of both enzymes which remains to be elucidated.

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REFERENCES

1. Chlapowski,F.J.,Kelly,L.A. and Butcher,R.W.(1975)in"Advances in cyclic nucleotide research"eds Greengard,P.and Robinson,G.A.(Raven Press,New York vol. 6, pp; 245-338.
2. Friedman,D.L., Johnson, R.A. and Zeilig,C.E. (1976)in "Advances in cyclic nucleotide Research" eds Greengard, P. and Robinson,G.A. (Raven Press, New York) vol. 7, pp.69-114.
3. Zeilig,C.E., Johnson,R.A., Sutherland,E.W. and Friedman,D.L.(1976) J.Cell Biol. 71,515-534.
4. Radley, J.M. and Hodgson,G.S.(1973) Exptl. Cell. Res. 80,237-244.
5. Zeilig, C.E. and Goldberg, N.D. (1977) Proc. Nat. Acad.Sci USA 74,1052-1056.
6. Franklin, T.J. and Foster, S.J. (1973) Nature 246,146-148.
7. Baird, W.M., Sedgwick, J.A. and Bootwell, R.K.(1971)Cancer Res.31,1434-1439
8. Lasne, C., Gentil,A., and Chouroulinkov,I.(1974) Nature 247, 490-491
9. Lowry, O.J. Rosebrough, N.J., Fan, A.L. and Randall, R.I.(1951) J. Biol. Chem. 193, 265-275.

10. Levilliers, J. , Pairault, J. Lecot,F., Laudat, M.H.(1976)FEBS Letters 63, 323-329
11. Rochette C., and Castagna M(1977)Biochm.Biophys.Res.Commun,74,1287-1296
12. Rochette-Egly, C., Chouroulinkov, I. and Castagna M. , submitted
13. Rochette-Egly, C., Chouroulinkov, I. and Castagna M., submitted
14. Millis, A.J.T., Forrest, G.A. and Pious,D.A.(1974) Exp. Cell.Res.83,335-343
15. Koide, Y., Earp, H.S., Ong,S.H. and Steiner,A.L.(1978) J.Biol.Chem.253, 4439-4445
16. Wells, J.N., Baird,C.E., Wu, Y.I. and Hardman, J.G. (1975) Biochim. Biophys. Acta 384, 430-442
17. Seifert,W.E., and Rudland,P.S.(1974) Proc. Natl. Acad.Sci. USA 70,4920-4924
18. Goldberg,N.D., Haddox,M.K., Dunham,E. Lopez,C. and Hadden J.W. (1973) in "the cold Spring Harbor Symposium on Regulation of Proliferation in Animals cells", eds Clarkson, B. and Baserga,R. (Cold Spring Harbor Lab. N.Y.) p. 609-625.