Vol. 111, No. 2, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 16, 1983 Pages 430-437

INTERFERON AFFECTS INTRACELLULAR CALMODULIN LEVELS

Marie-Françoise BOURGEADE, Françoise BESANÇON and Ming Nguy THANG

Institut de Biologie Physico-Chimique
13, rue Pierre et Marie Curie
75005 Paris - France

Received January 18, 1983

Summary : Interferon lowers calmodulin levels in two cell lines sensitive to its antiproliferative effect. Further, in synchronized cells, interferon strongly inhibits the increase in calmodulin observed when control cells enter the S phase, and concomitantly inhibits DNA synthesis. Calmodulin has been implicated in the control of cell proliferation and an increase in this protein seems to be necessary for the progression of cells into the S phase of the cell cycle. Therefore, the effect of interferon on calmodulin content might constitute part of the molecular mechanism by which interferon inhibits DNA synthesis.

Calmodulin, the major intracellular calcium receptor, has been demonstrated to mediate regulation by calcium of a large number of cellular activities. It binds and activates several enzymes (1-3) and plays an important part in controlling cell proliferation both in vitro and in vivo. An increase in calmodulin has been found as the cells synchronously enter the DNA synthetic phase of the cell cycle (3, 4, 5). In addition exogenously added calmodulin has the ability to stimulate DNA synthesis in isolated liver cells (6). The antiproliferative effect of interferon on many cell lines is well documented (7, 8) although the mechanism by which interferon reduces cell growth remains unclear. Some reports indicate that cells are blocked in the G_0-G_1 phase of the cycle after interferon treatment, and that interferon inhibits the transition from the G_1 to the S phase (9-12). However, others, using different cell lines showed that all the phases of the cell cycle are longer in cells treated with interferon (13). Since calmodulin has been implicated in cell proliferation, we attempted to ascertain whether interferon affects the amount of this protein present in cultured cells.

0006-291X/83 $1.50

MATERIAL AND METHODS

Cells.

Human Wish cells were grown either in Dulbecco medium supplemented with 10% foetal calf serum or in F_{12}/Dulbecco medium (V/V) supplemented with 5 µg/ml insulin, 5 µg/ml transferrin and 1 µg/ml Epidermal growth factor (EGF). Bovine MDBK cells were grown in Eagle medium supplemented with 10% newborn calf serum.

Interferon.

Interferon was induced by challenge of human lymphocytes with Sendaï virus and purified as previously described (14). The 21K fraction used for the experiments had a specific activity of 5.10^7 international units/mg of protein.

Chemicals.

Insulin, transferrin and trifluoperazine were purshased from Sigma Chemicals C.O., EGF from collaborative Research, [H^3] Thymidine (specific activity : 25 Ci/mmole) from C.E.A., France.

Calmodulin determination.

Cells were washed twice with cold Eagle medium, scrapped with a rubber policeman and centrifuged for 5 min. at 600g. The cell pellet was homogenized on ice by 30 strokes of Dounce in 0,5 ml of buffer containing 10 mM imidazole pH 7.5, 1 mM 2 β mercaptoethanol, 1 mM EGTA, 10 mM $MgCl_2$ and 0.15 M NaCl. Homogenates were heated for 6 min. at 80°C, rapidly cooled and centrifuged for 30 mn at 10000 g. Supernatants were assayed for calmodulin content using a [^{125}I] calmodulin radioimmuno assay kit from New England Nuclear (NEN). Protein content was measured by the Lowry method (15). Results are expressed in ng of calmodulin / mg of protein in the cell extract and represent the mean of two measurements. The intra-assay variability was less than 5%. The absolute values obtained in similar experiments varied due probably to the differences in the age of the cells (as it has been previously underlined by Chafouleas et al. (16)). Because of this interassay variability, data were not treated statistically.

DNA synthesis measurement.

Cells were pulse-labelled for 1 hour with 5 µCi/ml of [^{3}H] Thymidine ; they were then rinced three times with cold PBS, dissolved in 0,3% SDS, precipitated with 10% TCA and the radioactivity in the soluble and insoluble TCA fractions was counted. Results are the mean of 3 experiments.

RESULTS

Effect of interferon on calmodulin levels in exponentially growing cells.

To estimate an effect of interferon on calmodulin levels, exponentially growing bovine MDBK cells and human WISH cells cultivated in the presence of calf serum were treated either for 24 hours or two passages (i.e. 2 weeks) with interferon (500 IU/ml), and the calmodulin levels were estimated in cell extracts. The results of three experiments, reported in Table 1, express the calmodulin content per mg of protein in these extracts. In control

Table 1. Effect of interferon on calmodulin levels in Wish and MDBK cells.

INTERFERON treatment	MDBK cells			WISH cells		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
None	3257	2745	2972	1230	1969	1345
24 H	1578	1850	2224	ND	ND	1024
2 passages	571	ND	ND	930	1408	ND

Human WISH cells were routinely propagated in Dulbecco medium supplemented with 10% foetal calf serum and MDBK cells in Eagle medium supplemented with 10% newborn calf serum. For the experiment, 10^6 cells were seeded per 60 mm petri dishes in culture medium. After two days at 37° cells were placed in fresh, non supplemented medium and treated with 500 IU/ml of interferon. Cells were extracted 24 hours later. For cell treatment throughout 2 passages in the presence of interferon, 500 IU/ml of interferon was added to culture medium after each trypsinization. The same amount was again added 24 hours before cell extraction Calmodulin content was determined as described in Material and Methods. Results are expressed in ng of calmodulin/mg of protein in the cell extract and represent the mean of two measurements.

cells, these amounts correspond to about 250 ng of calmodulin per 10^6 MDBK cells, and 150 ng per 10^6 WISH cells, and are within the range of the calmodulin values reported by others in transformed cells of different origins (17, 18). Treatment with interferon decreased calmodulin levels in both cell lines. The reduction was more pronounced in MDBK cells, especially when they were maintained for two passages in the presence of interferon.

Effect of interferon on calmodulin levels in synchronized cells.

Since calmodulin has been implicated in the G_1-S progression of the cell cycle (4, 5, 17), these results prompt us to investigate the effect of interferon on the changes in calmodulin content during the stimulation of DNA synthesis in synchronized cells.

For such experiments, we used WISH cells cultivated in serum free medium supplemented with insulin, transferrin and epidermal growth factor (EGF). Such medium allow to eliminate the possibility that many ill-defined components of the serum used in culture medium could bind and/or inactivate calmodulin. WISH cells cultivated in such medium were synchronized by growth factor

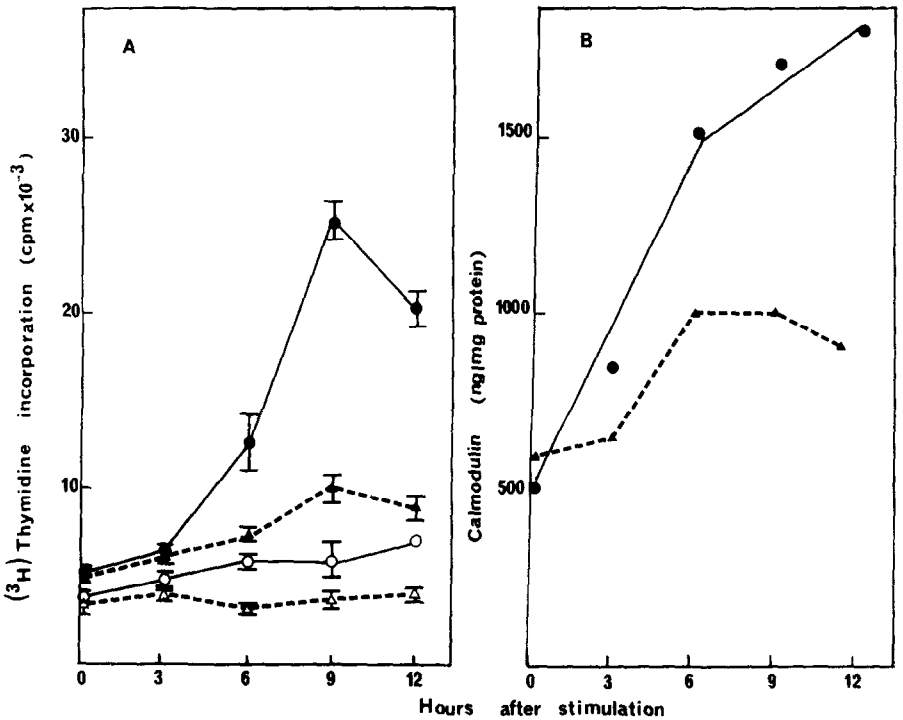

Figure 1. Effect of interferon on calmodulin levels and DNA synthesis in synchronized WISH cells.

WISH cells were routinely propagated in F12/Dulbecco (v/v) medium supplemented with 5 µg/ml insulin, 5 µg/ml transferrin and 1 ng/ml epidermal growth factor. For calmodulin determination, cells were seeded in F12/Dulbecco medium, in 60 mm petri dishes (10^6 cells/dish) and for measurement of DNA synthesis in 24 well-plates (10^5 cells/well) in the same medium. All cells were kept for one week in this medium without addition of growth factors. Stimulation was induced by adding fresh factor free medium. For interferon treatment, 500 UI/ml of human α interferon were added to the cells 24 hours before stimulation and the same amount was added again when stimulation started. At the times indicated, calmodulin content and DNA synthesis were estimated as described in Material and Methods. A, TCA insoluble radioactivity in control cells : •, in interferon treated cells : ▲ ; TCA soluble radioactivity in control cells : ○, in interferon treated cells : △ . B, calmodulin content in control cells : •, in interferon treated cells : ▲.

deprivation. Once the cells have been synchronized, their DNA synthesis could be stimulated at least for one cycle by adding fresh factor free medium.

DNA synthesis and calmodulin content were measured in control and in interferon treated cells at different times after the beginning of cell stimulation.

Results (Fig. 1) show that after such stimulation, there was a good correlation in control cells between their entry into the DNA synthesic phase

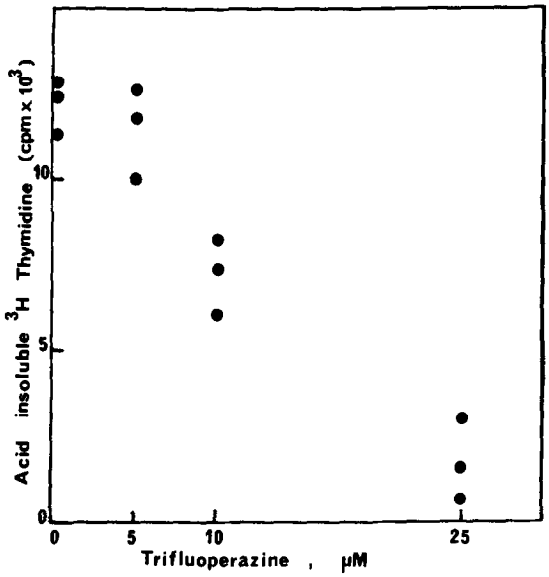

Figure 2. Effect of TFP on DNA synthesis in WISH and MDBK cells.

Exponentially growing WISH cells, cultivated in growth factor supplemented medium, were treated with TFP for 3 hours. DNA synthesis was then measured as described in Material and Methods.

and the rise in their calmodulin levels. In interferon treated cells, a parallel inhibition was observed in DNA synthesis and in the increase in the calmodulin content.

Inhibition of DNA synthesis by interferon was apparently not due to a change of cell permeability to thymidine since interferon treatment only slightly inhibited [^{3}H] Thymidine uptake in the acid-soluble pool, as previously reported (18). Since results are expressed as the amounts of calmodulin per mg of protein in cell extracts, inhibition of the increase in calmodulin was certainly not resulting of an inhibition of total protein synthesis by interferon, but rather seemed to be specific for calmodulin.

Effect of Trifluoperazine on DNA synthesis.

To further assess the correlation between the cellular content in calmodulin and the rate of DNA synthesis, we measured the effect of trifluoperazine (TFP), a calmodulin antagonist, on DNA synthesis in Wish cells (2). DNA synthesis is inhibited almost completly by TFP at 25 μM (Fig. 2). This result strenghtens the assumption that in Wish cells DNA synthesis might be closely

related to the calmodulin level. However, since side effects of the drug have been reported, the inhibitory effect of TFP on DNA synthesis might not be due only to its binding to calmodulin. Experiments using drugs with similar hydrophobicity and different affinities for calmodulin, such as W5 and W7 are under investigation.

DISCUSSION

Human α interferon reduces calmodulin levels in two different cell lines. In synchronized cells, interferon strongly inhibits the increase in calmodulin observed when control cells enter the S phase and concomitantly inhibits DNA synthesis. The inhibitory effect of TFP on DNA synthesis suggests that active calmodulin is necessary for the progression of our cells into the S phase. Therefore the effect of interferon on calmodulin content might constitute part of the molecular mechanism by which interferon inhibits DNA synthesis

In interferon treated cells, several enzymes are induced, including 2'5' oligoadenylate synthetase which catalyzes the synthesis of a series of oligoadenylates (2'5'A) characterized by a 2'5' phosphodiester linkage (19, 20). 2'5' oligoadenylates are potent inhibitors of protein synthesis in cell free systems and also when they are artifically introduced into intact cells where they activate an endonuclease which degrades mRNA (21, 22, 23). Using synchronized 3T3 cells, Kimchi et al. showed that like interferon, exogenously added 2'5' oligoadenylates reduced the number of cells entering the S phase (24). This suggest that activation of the 2'5' A system is involved in the antiproliferative effect of interferon. One of the mechanisms by which interferon regulates intracellular calmodulin content might be activation of the 2'5' A dependent endonuclease.

Recent studies have implicated microtubule depolymerization in the signal transduction for DNA synthesis (25-28). Calmodulin has been demonstrated to mediate the Ca^{++} directed depolymerization of microtubules in vitro (29-31). It was therefore suggested that the rise in calmodulin concentration at the G_1-S boundary should augment the proportion of depolymerized tubulin in the cell, leading to decreased tubulin synthesis. Such a decrease in tubulin

synthesis has, indeed, been observed during the S phase (32). In a recent report, Fellous et al. have shown that α and β interferon greatly increase the amount of tubulin mRNA in human lymphoblastoid cells (33). It is thus tempting to speculate that this increase might be a consequence of the interferon induced decline in calmodulin levels. In this connection, it would be worth finding out whether interferon alters the polymerization/depolymerization state of tubulin.

Various reports indicate that there is more calmodulin in spontaneously or virally transformed cells than in their normal counterparts (16, 34, 35). The present results showing that interferon reduced the calmodulin content of transformed cells constitute an additional demonstration of the ability of interferon to endow cancerous cells with a property inherent in normal cells.

ACKNOWLEDGMENT

This work was supported by Centre National de la Recherche Scientifique (E.R. 238), and Institut National de la Santé et de la Recherche Médicale (U. 245 and CRL 80-10-23).

REFERENCES

1. Means, A.R. and Dedman, J.R. (1980) Nature 285, 73-77.
2. Cheung, W.Y. (1980) Science 207, 19-27.
3. Means, A.R., Tash, J.S. and Chafouleas, J.G. (1982) Physiol. Reviews 62, 1-39.
4. Sasaki, Y. and Hidaka, H. (1982) Biochem. Biophys. Res. Comm. 104, 451-456.
5. Chafouleas, J.G., Bolton, W.E., Hidaka, H., Boyd III, A.E. and Means, A.R. (1982) Cell 28, 41-52.
6. Boynton, A.L., Whitfield, J.F. and Mac Manus, J.P. (1980) Biochem. Biophys. Res. Comm. 95, 745-749.
7. Gresser, I. (1977) Cellular Immunol. 34, 406-415.
8. Brouty-Boye, D. (1980) Lymphokine Reports 1, 99-112.
9. Sowaka, Y., Wantanabe, Y. and Kawade, Y. (1977) Nature 268, 236-238.
10. Creasey, A.A., Bartholomew, J.C. and Merigan, T.C. (1980) Proc. Natl. Acad. Sci. USA 77, 1471-1475.
11. Leanderson, T. and Lundgren, E. (1980) Exp. Cell. Res. 130, 421-426.
12. Taylor-Papadimitriou, J., Shearer, M. and Rozengurt, E. (1981) J. of Interferon Research 1, 401-409.
13. Killander, D., Lindahl, P., Lundin, L., Leary, P. and Gresser, I. (1976) Eur. J. Immunol. 6, 56-59.
14. Thang, M.N., Thang, D.C., Chelbi, M.K., Robert-Galliot, B., Commoy-Chevalier, M.J. and Chany, C. (1979) Proc. Natl. Acad. Sci. USA 76, 3717-3721.
15. Lowry, D.H., Rosebrough, N.J., Far, A.L. and Ravdall, R.J. (1951) J. Biol. Chem. 193, 265-275.
16. Chafouleas, J.G., Pardue, R.J., Brinkley, B.R., Dedman, J.R. and Means, A.R. (1981) Proc. Natl. Acad. Sci. USA 78, 996-1000.
17. Hidaka, H., Sasaki, Y., Tanaka, T., Endo, T., Ohno, S. Fujii, Y. and Nagata, T. (1981) Proc. Natl. Acad. Sci. USA 78, 4354-4357.
18. Tovey, M., Brouty-Boye, D. and Gresser, I. (1975) Proc. Natl. Acad. Sci. USA 72, 2265-2269.

19. Kerr, I.M., Brown, R.E. and Hovanessian, A.G. (1977) Nature 268, 540-542.
20. Baglioni, C. (1979) Cell 17, 255-264.
21. Williams, B.R.G., Golgher, R.R. and Kerr, I.M. (1979) FEBS Lett. 105, 47-52.
22. Hovanessian, A.G., Wood, J., Meurs, E. and Montagnier, L. (1979) Proc. Natl. Acad. Sci. USA 76, 3261-3265.
23. Drocourt, J.L., Dieffenbach, C.W., Ts'o, P.O.P., Justesen, J. and Thang, M.N. (1982) Nucl. Acids Res. 10, 2163-2174.
24. Kimchi, A., Shure, H., Lapidot, Y., Rapoport, S., Panet, A. and Revel, M. (1981) FEBS Lett. 134, 212-216.
25. Teng, M., Bartolomew, J.C. and Bissell, M.G. (1977) Nature 268, 739-741.
26. Friedkin, M., Legg, A. and Rozegurt, E. (1979) Proc. Natl. Acad. Sci. USA 76, 3909-3912.
27. Otto, A., Zumbe, A., Gibson, L. and Kubler, A.M. (1979) Proc. Natl. Acad. Sci. USA 76, 6435-6438.
28. Crossin, K.L. and Carney, D.H. (1981) Cell 23, 61-71.
29. Marcum, M.J., Dedman, R.J., Brinkley, B.R. and Means, A.R. (1978) Proc. Natl. Acad. Sci. USA 75, 3771-3775.
30. Jemiolo, K.K., Burgess, W.H., Rebhun, K.I. and Kreitsinger, R.H. (1980) J. Cell. Biol. 87, 248a.
31. Job, D., Fischer, E.H. and Margolis, R.L. (1981) Proc. Natl. Acad. Sci. USA 78, 4679-4682.
32. Klevecz, R.R. and Forrest, G.L. (1975) Ann. N.Y. Acad. Sci. 253, 292-303.
33. Fellous, A., Ginzburg, I. and Littaver, U.Z.(1982) The EMBO Journal 1, 835-839.
34. Watterson, D.M., Van Eldik, L.J., Smith, R.E. and Vanaman, T.C. (1976) Proc. Natl. Acad. Sci. USA 73, 2711-2715.
35. Mac Manus, J.P., Braceland, B.M., Rixon, R.H., Whitfield, J.F. and Morris, H.P. (1981) FEBS Lett. 133, 99-102.