Calcium Ionophore-induced Transient Down-regulation of c-myb mRNA Levels in Friend Erythroleukemia Cells*

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The effects of calcium ionophores A23187 and ionomycin on the c-myb and c-myc mRNA levels have been investigated in the Friend erythroleukemia cell line F4-6 using Northern blot analysis. Treatment of the cells with 0.5–4 μM A23187 or 1–4 μM ionomycin induced a concentration-dependent decrease in c-myb mRNA; this decrease was abolished by EGTA. c-myc mRNA levels were only moderately affected. After 12–24 h of calcium ionophore exposure, c-myb mRNA returned to pretreatment levels. No similar decrease in c-myb mRNA was seen with the sodium ionophore monensin (up to 16 μM). The dimethyl sulfoxide-induced suppression of c-myb and also of c-myc mRNA levels was not prevented in Ca2+-free medium and thus appeared Ca2+-independent. A23187 and ionomycin were capable of inducing β-globin mRNA synthesis in F4-6 cells. Prolonged calcium ionophore exposure, however, strongly reduced cell viability and resulted only in a slight hemoglobin increase at lower concentrations.

These results suggest that a rise in [Ca2+], may be a signal leading to a transient decrease in c-myb mRNA and the initiation of erythroid differentiation in Friend cells. The transient suppression of c-myb mRNA levels represents a common feature of the action of dimethyl sulfoxide and calcium ionophores.

Friend erythroleukemia cell lines derived from Friend virus-transformed mouse spleen cells (1, 2) have been used extensively to study the chemically induced differentiation. Friend cells grow indefinitely in suspension culture, but they can be induced to differentiate along the erythroid pathway when treated with dimethyl sulfoxide (Me2SO) or a variety of other inducing agents (3–5).

Despite a large number of studies, the precise mechanism by which these agents induce differentiation is still unresolved. Earlier experiments suggested that an increased Ca2+ influx might be an essential step in the initiation of differentiation in Friend erythroleukemia cells (6–11). This influx was thought to occur via a Na+/Ca2+ antiport system triggered by a decreased activity of the plasma membrane Na+K+-ATPase (9, 12). Although this hypothesis could not be confirmed in subsequent studies (13–15), these experiments suggest that Ca2+ influx caused by the calcium ionophore A23187 may have effects on the early cellular events involved in the induction of differentiation (6, 7, 9, 10). In fact, it has been demonstrated recently that A23187 as well as another chemically distinct calcium ionophore, ionomycin, can trigger commitment to differentiation in Friend cells (16).

Modulation of the expression of nuclear protooncogenes such as c-myc, c-myb, and c-fos, may be involved in the early cellular changes leading to differentiation in Friend cells (5). A characteristic transient decrease in c-myc and c-myb mRNA levels has been observed in Friend cells during the first few hours after treatment with the known chemical inducers of differentiation, Me2SO, hexamethylene bisacetamide (HMBA), and hypoxanthine (17–22) as well as with the potent differentiation-inducing anthracycline antitumor antibiotic, aclacinomycin A (23). However, the biochemical mechanisms underlying these rapid changes in the oncogene expression as well as their involvement in the commitment process have not been defined.

Since calcium ionophores induce early biochemical changes in Friend cells that may result in commitment to differentiation (16), the present experiments were undertaken to investigate whether calcium ionophores can produce early effects on the levels of c-myc and c-myb mRNAs in Friend erythroleukemia cells.

Materials and Methods

Chemicals—A23187, ionomycin, and monensin were purchased from Sigma. The ionophores were dissolved in ethanol. Experiments demonstrated that the resulting solvent concentration in the cell culture medium had no effect on the mRNA levels or cell differentiation.

[α-32P]dCTP (3000 Ci/mmol) was obtained from Amersham Buchler (Braunschweig, Federal Republic of Germany); a minimal essential medium without nucleosides, fetal calf serum, and penicillin/streptomycin solution (5000 units/ml penicillin and 5000 μg/ml streptomycin) were purchased from Gibco (Karlsruhe, FRG); analytical grade chemicals were from Sigma, Fluka (Buchs, Switzerland), and Merck (Darmstadt, FRG).

Cells and Culture Conditions—Friend erythroleukemia cells, line F4-6 (2), were kindly provided by Dr. W. Ostertag, Heinrich Pette Institute for Experimental Virology (Hamburg, FRG). Cells were grown in α-medium without nucleosides, supplemented with 10% fetal calf serum, 2 mM glutamine, and 1% penicillin/streptomycin solution at 37 °C in a humified atmosphere containing 5% CO2.

For the experiments, exponentially growing cells were plated at 6–7 × 10⁶ cells/ml. Approximately 16 h later, the cells were treated with the test substances, and at defined time points thereafter cells were harvested and stored at –80 °C before RNA isolation. To measure hemoglobin production, cells were incubated for 4 days after treatment, and hemoglobin content of the cells was determined by the benzidine technique of Luftig et al. (24) using bovine hemoglobin as standard. Cell viability was examined by trypan blue exclusion.

Northern Blot Analysis—Total cellular RNA from F4-6 cells was isolated by the acid guanidinium thiocyanate-phenol-chloroform method.
method of Chomczynski and Sacchi (25). 30 μg samples of RNA were
denatured by glyoxylation, size-separated by electrophoresis
through 1% agarose gel, and transferred to Biodyne A membrane
(Pall, Portsmouth, United Kingdom) by the capillary blotting tech-
nique (26) using 20 × standard saline citrate solution (SSC, 1 × SSC
contains 0.15 M sodium chloride and 0.015 M sodium citrate). RNA
was immobilized by baking the membrane at 80 °C for 1.5 h. The
cDNA probes for hybridization were labeled with [α-32P]dCTP by the
random primer method (27) using the multiprime DNA-labeling
system from Amersham Buchler (Braunschweig, FRG). Membrane
prehybridization (6 h) and hybridization (16–18 h) were carried out
at 42 °C in 50% (c-myc, β-actin, and β-globin) or 40% (v-myb)
formamide, 50 mM sodium phosphate buffer, pH 6.5, 5 × SSC, 5 ×
Denhardt’s solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1%
polyvinylpyrrolidone), 0.1% SDS, and 250 μg/ml denatured herring
sperm DNA (Boehringer, Mannheim, FRG). Hybridized blots were
washed initially in 2 × SSC containing 0.1% SDS at room temperature
for 30 min and at 50 °C twice for 30 min. More stringent washing
was performed after hybridization with c-myc and β-actin cDNA
probes in 0.2

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SSC and 0.1% SDS at 55 °C twice for 30 min, after
hybridization with β-globin cDNA in 0.1 × SSC and 0.1% SDS at
55 °C twice for 30 min and after hybridization with v-myb in 0.5 ×
SSC and 0.1% SDS at 50 °C twice for 30 min. The membranes were
exposed at −80 °C to an x-ray film using intensifying screens. Follow-
ing stripping in 50% formamide and 10 mM sodium phosphate buffer,
pH 6.5, at 65 °C for 30 min, the membranes were subsequently
hybridized with the cDNA probes.

Hybridization probes were as follows: human β-actin cDNA, 1.2-
kb PstI fragment kindly provided by Dr. T. Braun (Institute of
Biochemistry and Biotechnology, University of Braunschweig, FRG); mouse β2-microglobulin cDNA (28, 29) was generously donated by Dr. J.
Nowock (Heinrich Pette Institute for Experimental Virology, Ham-
burg, FRG). The 6.2-kb EcoRI-PstI genomic fragment containing the
full β2-microglobulin gene (28) was used; human c-myc exon 3 (1.4 kb)
and v-myb (0.8 kb) were purchased from Oncor (Gaithersburg, MD).

RESULTS

Effect of A23187 on the c-myc and c-myc mRNA Levels—
F4-6 cells were exposed to the calcium ionophore A23187 at
the concentrations of 0.5, 1, 2, and 4 μM. Three h after
treatment the cells were harvested, and c-myc and c-myc mRNA
levels were measured using Northern blot analysis (Fig. 1). The levels of β-actin mRNA were also determined as
an internal control for RNA loading. The results show a
concentration-dependent decrease in c-myc mRNA
levels after A23187 treatment. The c-myc mRNA levels were only
moderately affected at the two highest concentrations tested.
The effect of A23187 was studied also in the presence of
EGTA, 3 mM (Ca2+ concentration in the medium used was
~2.1 mM). The results demonstrate that the effect of A23187
on the c-myc expression was abolished in the presence of
EGTA. This indicates that the observed effect of A23187,
indeed, is depending on the presence of extracellular Ca2+.

To further characterize the effect of A23187, 1 μM, on
the c-myc mRNA levels, a time course analysis was performed
over a treatment period of 12 h. As shown in Fig. 2, c-myc
mRNA levels began to decrease at 2 h after treatment. c-myc
mRNA levels showed a minimum at 3–6 h. After 9 h, c-myc
mRNA levels recovered and approached pretreatment levels
at 12 h.

Effect of Ionomycin on the c-myc and c-myc mRNA Levels—
In further experiments the effect of ionomycin, another cal-
cium ionophore, was investigated on c-myc and c-myc mRNA
levels in F4-6 cells. Fig. 3 shows the effect of ionomycin at
concentrations of 1, 2, and 4 μM and 1 and 3 h after treatment
and at concentrations of 1 and 2 μM 24 h after treatment.
Similarly to the results obtained with A23187, ionomycin
induced a concentration-dependent decrease in c-myc mRNA
levels 3 h after drug exposure. No changes in the levels of c-
myb mRNA could be observed 24 h after treatment as com-
pared with the untreated control. c-myc mRNA levels re-
main unchanged or only slightly affected during incubation
with ionomycin. As shown in Fig. 4, the effect of ionomycin,
2 μM on the c-myc mRNA levels was antagonized in the
presence of EGTA, 3 mM.

Effect of Monensin on the c-myc and c-myc mRNA Levels—To
characterize the specificity of the action of calcium ionophores
on the c-myc expression, we studied the effect of the sodium

![Fig. 1. Changes in the levels of c-myc mRNA and c-myc mRNA in F4-6 cells after a 3-h exposure to A23187 (0.5, 1, 2, and 4 μM) in the presence and absence of EGTA (3 mM). Total cellular RNA was isolated and tested using Northern blot analysis. Amounts of 30 μg of denatured RNA were size fractioned on 1% agarose gel and transferred by the capillary blotting technique to a nylon filter. Blots were hybridized sequentially with v-myb, third exon c-myc, and β-actin cDNA probes. β-Actin mRNA was determined as a control to verify the amount of RNA in each lane.](image1)

![Fig. 2. Time course-dependent effect of A23187 (1 μM) on c-myc, c-myc, and β-actin mRNA levels in F4-6 cells. Cells were exposed to A23187 for the entire time course of the experiment. For further experimental conditions see Fig. 1.](image2)

![Fig. 3. c-myc, c-myc, and β-actin mRNA levels in F4-6 cells treated with ionomycin (1, 2, and 4 μM) for 1, 3, and 24 h. For experimental conditions see Fig. 1.](image3)
ionophore monensin on the c-myb and c-myc mRNA levels in F4-6 cells. As A23187, monensin acts as a H⁺ exchanger, leading to an increase in cytoplasmic pH. This has also been discussed as being responsible for the effects of A23187 in Friend erythroleukemia cells (15). The results showed that monensin, studied at the same concentrations as the calcium ionophores (1-4 μM) or higher (up to 16 μM) did not induce a decrease in c-myb mRNA levels (Fig. 5). Moreover a slight increase in c-myb and a decrease in c-myc transcript levels could be observed in F4-6 cells after 3 h of treatment with monensin.

Effect of EGTA on the Me2SO-induced Changes in c-myb and c-myc mRNA Levels—The results with the two calcium ionophores suggest that Ca²⁺ influx in Friend erythroleukemia cells can induce a transient decrease in c-myb mRNA levels. Because chemical inducers of the differentiation, such as Me₂SO, are known to induce similar changes (19–23), we studied the effect of EGTA, 3 mM, on the Me₂SO-induced decrease in c-myb and c-myc transcript levels (Fig. 6). In accordance with previous data (17–23), Me₂SO, 1.5%, induced a strong decrease in c-myc and c-myc mRNA levels within 1 and 3 h, respectively. These changes were not antagonized by the presence of EGTA in the incubation medium. A slight increase in c-myb (at 3 h) and decrease in c-myc mRNA levels was observed with EGTA alone. Similar negative results were obtained when the cells had been incubated in Ca²⁺ free medium (3 mM EGTA) for 18 h before Me₂SO treatment (Fig. 7A) or when the EGTA concentration was raised from 3-5 or 10 mM (Fig. 7B). These results strongly suggest that the Me₂SO-induced suppression of c-myb expression occurs in a Ca²⁺-independent manner.
were incubated in drug-free medium for additional period of treatment with the calcium ionophores, cell viability was reduced at concentrations below 0.5 μM. The sodium ionophore monensin, tested up to 16 μM (Table 1). A23187 and ionomycin have been shown to induce β-globin mRNA synthesis in the early phase of treatment, which was measured 24 h after drug removal (Fig. 8), consistent with these results. It should be noted, however, that Friend cells can be induced to synthesize globin mRNA also in the absence of commitment (5). Although the increased β-globin mRNA expression indicates that the cells were executing the terminal differentiation program, longer incubation of F4-6 cells with A23187 and ionomycin strongly reduced cell viability and thus only slight increases in hemoglobin production could be demonstrated at lower concentrations (Table 1). It is noteworthy that the changes in c-myb mRNA levels after calcium ionophore treatment are similar to those after exposure of Friend cells to chemical inducers of differentiation (19-23). On the other hand, the rapid transient decrease in c-myc expression, which is observed after treatment with Me2S0 and other inducers (17-23), was not seen with the calcium ionophores. These results may point to the importance of the decrease in c-myb expression for the early cellular events of Friend cell differentiation.

In contrast, expression of transfected c-myb CDNAs during the early phase of inducer treatment does not block differentiation or early commitment effects in Friend cells (31, 32). It has been suggested that the postcommitment down-regulation of c-myb mRNA at a later time, not the early transient decrease, is related to terminal differentiation. However, it is important to consider that the expression of transfected c-myb in Friend cells does not prevent the early effects of Me2S0 or HMBA on the endogenous c-myb and c-myc mRNA regulation (31, 33). Therefore, these experiments do not exclude the possibility that the early down-regulation of endogenous c-myb expression is involved in the commitment program. Furthermore, in c-myc-transfected Friend cells, the levels of exogenous c-myc protein synthesis are decreased by up to 50% or more upon exposure to Me2S0 during the commitment period (22). The question arises whether in c-myb-transfected cells a similar decrease in c-myb protein synthesis occurs during the commitment phase, in spite of the high levels of exogenous c-myb mRNA (31-33).

The effect of Me2S0 on c-myb and c-myc mRNA levels was
not prevented by the presence of EGTA (Figs. 6 and 7). Accordingly, the MeSO-induced decrease in c-myb mRNA levels appears independent of Ca2+. This observation is consistent with previous results showing that MeSO causes a slight decrease in cytosolic Ca2+ concentration (15, 16). Therefore, MeSO and calcium ionophores decrease c-myb mRNA levels via different mechanisms. In addition, the effect of MeSO on c-myb mRNA levels, but not that of the calcium ionophores is accompanied by a similar effect on the c-myb expression (19, 21–23). However, c-myb mRNA levels may represent a common target of the action of MeSO and calcium ionophores in Friend erythroleukemia cells.

Erythropoietin-induced differentiation in SKT6, J2E, and Rauscher mouse erythroleukemia cells has been shown to lead to a decline in c-myb mRNA levels and an increase in c-myc transcripts in the early stage of differentiation (34–36). On the other hand, MeSO or sodium butyrate treatment results in a decrease of both c-myb and c-myc mRNA levels in these cells. It was concluded that down-regulation of c-myb gene expression is an important prerequisite for either erythropoietin initiated or chemical-induced erythroid differentiation (34, 35). The early transient down-regulation of c-myb mRNA levels in J2E cells by erythropoietin (35) showed a similar kinetic to that observed with calcium ionophores in the present experiments (Fig. 2). Earlier studies demonstrated that calcium ionophores enhance colony formation by erythroid colony-forming units and that EGTA inhibits such colony formation (37). A series of subsequent studies suggest that calcium may be involved in the erythropoietin-mediated signal transduction, although there are also conflicting results (reviewed in Ref. 38).

Considering our present results, therefore, the question arises whether the early down-regulation of c-myb expression by erythropoietin is mediated by a calcium signal. In this case, the effect of calcium ionophores on c-myb expression shown in the present experiments may represent a part of the erythropoietin signal pathway in erythroid precursor cells. The stimulation of c-myc expression by erythropoietin (34–36) has been shown to occur through other mechanisms (36) and may also be related to the cell proliferation-stimulating activity of erythropoietin (35, 39). Further experiments with erythropoietin responsible cell lines are necessary to reveal this possibility. However, if the erythropoietin-induced decline of c-myb expression occurs in a Ca2+-independent manner, our results with calcium ionophores would represent another alternative mechanism of c-myb mRNA down-regulation with inducers of the commitment to terminal differentiation (16) and would still underline the importance of the regulation of c-myb expression in the early phase of erythroid differentiation.

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**REFERENCES**

1. Friend, C., Scher, W., Holland, J. G., and Sato, T. (1971) Proc. Natl. Acad. Sci. U. S. A. 68, 379–382
2. Osterburg, W., Melderis, H., Steinheider, G., Kluge, N., and Dube, S. (1972) Nature 239, 325–334
3. Marks, P. A., and Rikkind, R. A. (1978) Annu. Rev. Biochem. 47, 419–448
4. Reuben, R. C., Rikkind, R. A., and Marks, P. A. (1980) Biochem. Biophys. Acta 600B, 325–346
5. Marks, P. A., Sheffley, M., and Rikkind, R. A. (1987) Cancer Res. 47, 659–668
6. Levenson, R., Houseman, D., and Cantley, L. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 9948–9952
7. Bridges, K., Levenson, R., Houseman, D., and Cantley, L. (1981) J. Cell. Biol. 90, 542–544
8. Tsiftsoglou, A. S., Mitrani, A. A., and Houseman, D. E. (1981) J. Cell. Physiol. 106, 327–335
9. Smith, R. L., Macara, I. G., Levenson, R., Houseman, D., and Cantley, L. (1983) J. Biol. Chem. 257, 773–780
10. Levenson, R., Macara, I. G., Smith, R. L., Cantley, L., and Houseman, D. (1982) Cell 28, 555–565
11. Levenson, R., Macara, I. G., Cantley, L., and Houseman, D. (1983) J. Cell. Biochem. 21, 1–8
12. Yeh, L.-A., Ting, L., English, L., and Cantley, L. (1983) J. Biol. Chem. 258, 6567–6574
13. Schaefer, A., Munter, K.-H., Heck, P., and Koch, G. (1984) J. Cell. Physiol. 119, 335–340
14. Lannigan, D. A., and Knaut, P. A. (1985) J. Biol. Chem. 260, 7322–7324
15. Faletto, D., and Macara, I. G. (1985) J. Biol. Chem. 260, 4884–4889
16. Hensold, J. O., Dubyk, A., and Houseman, D. E. (1991) Blood 77, 1362–1370
17. Lachman, H. M., and Skoultchi, A. I. (1984) Nature 310, 249–251
18. Lachman, H. M., Hatton, K. S., Skoultchi, A. I., and Schildkraut, C. L. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5223–5227
19. Kirsch, I. R., Berthens, V., Silver, J., and Hollis, G. F. (1986) J. Cell. Biochem. 22, 11–24
20. Ramsay, R. G., Ikeda, K., Rikkind, R. A., and Marks, P. A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6849–6853
21. Watson, R. J. (1978) Mol. Cell. Biol. 8, 3934–3942
22. Spots, G. D., and Harn, S. R. (1986) Mol. Cell. Biol. 6, 3952–3964
23. Schaefer, A., Dressel, A., Lingelbach, K., Schmidt, C. A., Steinheider, G., and Marquardt, H. (1992) Leukemia 6, 838–833
24. Luftig, R. B., Conscience, J.-F., Skoultchi, A., McMillan, P., Revel, M., and Rodde, P. H. (1977) J. Biol. Chem. 252, 799–811
25. Chomczynski, P., and Sacchi, N. (1987) Anal. Biochem. 162, 156–159
26. Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5201–5207
27. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13
28. Tiede, D. C., Tillhagen, S. M., Polsky, P. I., Swolin, J. G., Leder, A., Marshall, H., and Leder, I. (1976) Cell 14, 337–345
29. Konkel, D. A., Mainzer, J. V., and Leder, P. (1979) Cell 18, 865–873
30. Sheng-Ono, G. I. C. (1990) Biochem. Biophys. Acta 1032, 29–52
31. McLaren, D., Stafford, J., Breuts, L., Bender, T. P., and Kuehl, W. J. (1990) Mol. Cell. Biol. 10, 765–770
32. Danish, R., El-Awar, O. Weber, R. L., Langmore, J., Turko, L. A., Ryan, J. J., and Clarke, M. F. (1992) Oncogene 7, 901–907
33. Clarke, M. F., Kukowska-Latalio, J. F., Westin, E., Smith, M., and Prochownik, E. V. (1988) Mol. Cell. Biol. 8, 884–892
34. Todokoro, K., Watson, R. J., Iigo, H., Anamura, H., Kuramochi, S., Yanagisawa, H., and Ikawa, Y. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8909–8914
35. Busfield, S. J., and Klinken, S. P. (1982) Blood 60, 412–419
36. Patel, H. R., Choi, T.-S., and Syrkowski, A. J. (1992) J. Biol. Chem. 267, 21290–21292
37. Minis, J., and Spivak, J. L. (1979) J. Clin. Invest. 64, 1573–1579
38. Spivak, J. L. (1992) Exp. Hematol. 20, 283–285
39. Syrkowski, A. J., Salvado, A. J., Smith, G. M., McIntyre, C. J., and DeBooth, N. J. (1986) Science 230, 74–76